Role of Osteopontin During Dextran Sulfate Sodium-induced Colitis

by

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For the degree of Doctor of Philosophy,
Graduate Department of Dentistry,
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ABSTRACT

Thesis title: Role of Osteopontin during DSS-induced Colitis
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Osteopontin (OPN) is a matricellular cytokine found in most tissues and body fluids. It is involved in a variety of cell processes by binding to integrins and CD44 receptors, and it modulates immune responses. To investigate the functions of OPN during colitis the DSS acute colitis model in OPN-/- and WT control mice was utilized. OPN-/- mice were more susceptible to DSS-induced colitis than the DSS-treated WT control mice. The increased susceptibility of the OPN-/- mice was characterized by greater intestinal crypt destruction; high myeloperoxidase activity of infiltrating neutrophils; lack of differentiation of inflammatory cells such as lymphocytes subsets (CD4+, CD8+) and macrophages (F4/80); reduced production of certain cytokines, especially TNF-alpha; and non-programmed cell death of enterocytes. It is postulated that the hyperactivity of neutrophils may explain the increased tissue destruction during experimental colitis in the absence of OPN. Analysis of OPN’s impact on neutrophil function showed that while OPN may be important for the recruitment and migration of neutrophils, the expression of OPN by neutrophils is not required for manifestation of their destructive capabilities. This would suggest that OPN administration may protect the intestines from the adverse effects of colitis. Exogenous bovine milk OPN (20 µg/ml), administered for 8 days dissolved in the drinking water, ameliorated DSS-induced colitis. It diminished signs of disease, with a greater impact in the WT than the OPN-/- mice. It reduced the levels of neutrophils, macrophages, and pro-inflammatory mediators in the colon tissue. Recombinant OPN failed to reproduce the beneficial effects of milk OPN, which suggests
that post-translational modifications of OPN are crucial to ameliorate experimental colitis. Collectively, these studies demonstrate that OPN has a protective effect during experimental colitis and that the oral administration of bovine milk OPN (20 µg/ml) ameliorates acute DSS-induced colitis. The results of this study also imply that the protective effect probably depends on a post-translationally modified form of OPN, and may require intracellular-OPN as a cofactor for significant attenuation of colitis. Future research could concentrate on more detailed investigation of these latter findings to determine OPN’s mechanism of action.
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THEESIS FORMAT

This PhD thesis is presented in the publishable style. Each of the chapters describing hypothesis testing has been either published or submitted for publication (chapter 5), and is presented in either the original published or manuscript form. An introduction and thesis summary are included to contextualize the experimental results with our current knowledge. The journals holding the copyrights for the papers published from this thesis have given permission for the reproduction of the text and figures for this thesis.
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Peer Reviewed Publications


* I was instrumental in the conception of this paper, assembling the literature and contributed to the writing.

+ I participated in discussions with Dr. Sodek and Dr. Zohar that led to the conception, contributed to preliminary experiments, and contributed by reviewing the final draft before submission.
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ABBREVIATIONS

5-ASA ..................5-aminosalicylic acid
AOM ..................azoxymethane
APC ..................antigen-presenting cells
b-OPN ..................biotinylated bovine milk OPN
BSA ..................bovine serum albumin
CD ..................Crohn's disease
DAI ..................disease activity index
DNBS ..................dinitrobenzenesulfonic acid
DSS ..................dextran sulphate sodium
Eta-1 ..................T-lymphocyte activation protein
EAE ..................experimental autoimmune encephalomyelitis
Fas ..................FS-7 cell associated cell surface antigen
fMLP ..................N-formyl methionyl leucyl phenylalanine
GALT ..................gut-associated lymphoid tissue
G-CSF ..................granulocyte colony-stimulating factor
GM-CSF ..................granulocyte-macrophage colony-stimulating factor
HLA ..................human leukocyte antigen
HRP ..................horseradish peroxidase
HSP-60 ..................heat shock protein 60
IBD ..................inflammatory bowel disease
IFN-γ .................interferon-γ
IL ..................interleukin (IL-1,2,4,5,6,7,8,10,12,13,17,18,23 all mentioned in thesis)
IgA ..................immunoglobulin A
IgG ..................immunoglobulin G
LPS ..................lipopolysaccharide
MALT ..................mucosal-associated lymphoid tissues
MAPK ..................mitogen-activated protein kinase
MCP-1 ..................monocyte chemoattractant protein-1
M-CSF ..................macrophage colony-stimulating factor
MHC ..................major histocompatibility complex
MIG .................... monokine induced by gamma-interferon
MMPs ............... matrix metalloproteinases
MPO .................. myeloperoxidase
NF-kB ................ nuclear factor kB
NKT-cells .......... natural killer T cells
NO ..................... nitric oxide
NSAID .............. non-steroidal anti-inflammatory drug
OPN .................... osteopontin
PAMPs .............. pathogen-associated molecular patterns
PBS .................. phosphate-buffered saline
PCD ............... programmed cell death
PMA ........... phorbol 12-myristate 13-acetate
PMNs ............. polymorphonuclear leucocytes
PRR ................ pathogen recognition receptors
r-OPN .............. recombinant OPN
RAG ................ recombination activating gene
RANTES .............. regulated on activation normal T cell expressed and secreted
RBC ................ red blood cells
RGD ................ arginine-glycine-aspartate
ROS ................ reactive oxygen species
SCID ............... severe combined immunodeficiency
α-SMA .......... α-smooth muscle actin
TCR ............... T cell receptor
TGF-β1 .............. transforming growth factor beta 1
Th1 ................... T helper 1
Th2 ................... T helper 2
Th17 .................. T helper 17
TLR ................ toll-like receptor
TNBS .............. trinitrobenzene sulfonate
TNF-α .......... tumor necrosis factor-alpha
Treg ................ regulatory T cell
UC ....................ulcerative colitis
VCAM-1 ..........vascular cell adhesion molecule-1
WBC ..............white blood cell
WT .................wild-type
CHAPTER 1: Introduction
Inflammatory Bowel Disease (IBDs)

Types and characteristics of IBD

In humans, the most common IBDs are ulcerative colitis (UC) and Crohn's disease (CD). Accounting for far fewer cases are other forms of IBD: collagenous colitis, lymphocytic colitis, ischemic colitis, diversion colitis, behçet's syndrome, infective colitis and indeterminate colitis (Geboes, 2008). Patients with CD possess a patchy inflammation in any part of the gastrointestinal tract, from mouth to anus. In contrast, ulcerative colitis presents as a diffuse mucosal inflammation restricted to the colon and the rectum (Carter et al, 2004; Bernstein et al, 2006). Another difference is that, microscopically, UC is restricted to the mucosa, while CD affects the whole bowel wall (Goodman et al, 1976). Ultimately, IBD is associated with a variety of extra-intestinal manifestations such as liver problems, arthritis, skin manifestations and eye problems. These problems may produce greater morbidity than the underlying intestinal disease and may even be the initial presenting symptoms of the IBD (Ardizzone et al, 2008).

Epidemiology

The prevalence of UC has increased in the past few decades in North America and Europe. It afflicts up to 1 million people in the U.S.A. (Terry et al, 2008), approximately 0.5% of the population of Canada (Bernstein et al, 2006), and it also appears to be increasing in prevalence in other parts of the world (Loftus, 2004). CD has a similar distribution but to a lesser degree. Notably, the incidence and prevalence of UC and CD are beginning to stabilize in high-incidence areas, such as northern Europe and North America. In contrast, they continue to rise in low-incidence areas, such as southern
Europe (Tragnone et al, 1996), Asia (Sood et al, 2003), and much of the developing world (Linares de la Cal et al, 1999). UC can occur in people of any age, but it usually starts between the ages of 15 and 30 (Loftus et al, 2000). It affects men and women equally and appears associated with inheritable genetic traits, including specific human leukocyte antigen (HLA) haplotypes (Rodriguez-Bores et al, 2007). Previously noted racial and ethnic differences seem to be narrowing. A higher incidence of UC is seen in Caucasians and people of Jewish descent (Odes et al, 1994). Several studies suggest that the incidence of IBD among African Americans is approaching that of Caucasians (Sawczenko et al, 2001). Studies of migrant populations suggest that ethnic and racial differences may be more related to lifestyle and environmental influences than truly genetic differences (reviewed in Loftus, 2004). Approximately 15% of patients with UC develop at least one acute attack of severe colitis, and 30% of these patients require colectomy.

**Symptoms and diagnosis**

The correct diagnose between UC and CD can be rather difficult. Both diseases may present with any of the following clinical symptoms: abdominal pain, vomiting, diarrhea, hematochezia, weight loss (Carter et al, 2004) and various associated complaints or diseases like arthritis, pyoderma gangrenosum, and primary sclerosing cholangitis. In UC, bloody stool, diarrhea and abdominal pain tend to be the major symptoms associated with slight anemia and signs and laboratory data consistent with inflammation (Yoshida and Yonezawa, 1999). As a consequence, patients with UC tend to have increased risks of various conditions, including nutrient deficiencies, liver/gall
bladder disease, kidney stones, osteoporosis, and colon cancer, depending on the duration and extent of the disease (Collins and Rhodes, 2006). The presence of disease in UC and assessment of disease activity is possible with the use of sigmoidoscopic visualisation with direct histopathological biopsy examination, as the disease is uniform and begins distally. This is not possible in CD due to its variable location and the patchy nature of the inflammation (Tibble et al 2000).

Rarely, a definitive diagnosis between CD or UC can be made because of idiosyncrasy in the presentation. There is no single "gold standard" test or examination to diagnose, to assess severity and to predict the outcome of IBD. Instead, physicians apply a combination of symptoms, clinical examination, laboratory indices, radiology, and endoscopy with histology (Huett and Xavier, 2008). Clinical indices give only indirect measurement of disease activity and may not accurately predict inflammatory activity found by endoscopic and histological examination. Endoscopy is accurate but is invasive and expensive. Therefore, there is a need to use alternate diagnostic tools to identify IBD.

**IBD- serum biomarkers**

Although diagnoses of IBD rely mostly on clinical symptoms, with a contribution from demographic data, such as smoking status, ancestry, and age (Huett and Xavier, 2008), there are a number of laboratory markers that could possibly contribute to the diagnosis of IBD (Vermeire et al 2006). Some of the methods widely used and easily applied for measuring intestinal inflammation are the serologic assessment of erythrocyte sedimentation rate, C reactive protein, $\alpha_1$-antitrypsin, orosomucoid ($\alpha_1$-acid glycoprotein), neutrophil elastase, platelet counts, haemoglobin, mean platelet volume,
serum thrombopoietin, and serum erythropoietin (Andre et al, 1981; Gabay and Kushner, 1999; Vermeire et al, 2004; Henriksen et al, 2008). Other specific serologic markers previously believed to differentiate CD from IBD, are the antibodies to baker's and brewer's yeast, anti-Saccharomyces cerevisiae antibody (ASCA), that have been described in patients with CD (Lecis et al, 2002; Oshitani et al, 2003). However, assessment of these serological markers have in general been disappointing because of their lack of sensitivity and specificity, they are only indirect measures of inflammation, and they can be affected by a number of nonintestinal diseases (Bruining and Loftus, 2006; Bruining and Loftus, 2007).

**IBD- cytokines biomarkers**

The expression of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, is markedly increased in the intestinal mucosa in patients with active IBD, although not always accompanied by increased concentration of this cytokine in the serum (Hyams et al, 1991). The same conclusion applies to the measurement of TNF-α concentration in stool (Saiki et al 1998). A number of other cytokines have been studied with the aim to find possible biomarkers for IBD, such as interleukin-1 (IL-1) (Propst et al, 1995) and soluble interleukin 2 (IL-2) receptor (Nielsen et al, 1993), interleukin 6 (IL-6) (Mahida et al, 1991), interleukin 10 (IL-10) (Kucharzik et al, 1995), interleukin 12 (IL-12)p40, and transforming growth factor beta 1 (TGF-β1) (Kader et al, 2005). Although these cytokines seem to be promising markers, further studies need to be done.

**IBD-faecal biomarkers**
Faecal diagnostic biomarkers for IBD can be divided into faecal excretion of leucocytes, serum proteins or leucocyte products. One of the promising markers is calprotectin, a calcium-binding protein found in neutrophils, monocytes, and macrophages. It comprises up to 60% of the total cytosolic protein content of neutrophils (Roseth et al, 1997), it resists metabolic degradation, and it can be measured in faeces (Roseth et al, 1992). Its use has been extensively validated, showing consistent abnormalities in patients with IBD (Roseth et al 1997). Another faecal marker useful in the assessment of the disease activity of IBD is lactoferrin (Langhorst et al, 2005), having similar sensitivity and specificity to calprotectin (D'Inca, 2007).

**IBD immunopathology**

The normal intestinal mucosal immune system is constantly stimulated by microbial and dietary antigens. Peptidoglycan, lipopolysaccharides, bacterial flagellin, lipoteichoic acid from gram-positive bacteria, nucleic acid variants normally associated with viruses, and metabolites of the diverse microbiota of the gastrointestinal tract activate and induce subsequent mucosal immunologic and inflammatory events. Complex components and solutes that display microbe-associated molecular patterns cross epithelial barriers and are taken up by antigen-presenting cells. Many are capable of activating macrophages and lymphocytes to release potent proinflammatory cytokines. Migration of innate immune cells, such as neutrophils, macrophages, and dendritic cells, into target mucosal tissues depends on the expression of cytokines, chemokines and adhesion molecules (reviewed in Xavier & Podolsky, 2007). The secreted mediators, including IL-1, IL-6, and TNF-α increase the presence of human leukocyte antigen
(HLA) class II antigen-presenting molecules on the surfaces of epithelial cells, endothelial cells, macrophages, and B cells, thus augmenting their ability to present lumenal antigens and bacterial products which stimulate naïve CD4+ T cells. Microorganisms and the cytokines IL-1 and TNF-α also stimulate epithelial cells, endothelial cells, macrophages, and fibroblasts to secrete potent chemotactic cytokines, such as interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1), which would increase migration of macrophages and granulocytes from the circulation into the inflamed mucosa. Moreover, activated T cells differentiate into T helper 1 (Th1) cells which release IL-2, IL-8, IL-12, interferon-γ (IFN-γ), nitric oxide (NO), free radicals, osteopontin (OPN) and TNF-α, which further activate Th1 cells and promote recruitment of neutrophils (Kosiewicz et al, 2001; Neurath et al, 2002). TNF-α, IL-12 and oxygen radicals produced by activated macrophages contribute to epithelial ulceration, resulting in further secretion of TNF-α and IL-8, which amplify the inflammatory response (Orlando, 2002). Persistent Th1 cells and neutrophils are normally cleared by apoptosis (Shetty and Forbes, 2002). However, during IBD, they appear to survive longer than Th2 cells through stimulation by IL-2, TNF-α, CD44, and possibly by OPN (Wittig et al, 2000; Gassler et al, 2002). Further, it has been shown that the total number of macrophages is increased. Subpopulations of macrophages that are not normally present in the lamina propria of the intestine appear (Hume et al, 1987; Oshitani et al, 1996), indicating ongoing cellular recruitment to the inflamed bowel (Rugtveit, 1994; Grimm et al, 1995). Consequently, macrophages may contribute to intestinal damage by releasing reactive oxygen species (ROS) (Britigan et al, 1988) and by secreting proinflammatory
cytokines and other inflammatory mediators, by phagocytosing foreign antigens or invasive micro-organisms, or by acting as antigen-presenting cells.

In order to terminate an inflammatory process, granulocytes need to be eliminated and the tissue population of macrophages and lymphocytes returned to normal pre-inflammation density and phenotypes (Gallin et al, 1999, reviewed in Serhan and Savill, 2005). Neutrophil apoptosis triggers recognition by macrophages, which is critical for the uptake of neutrophils by macrophages. This is an essential step in inflammation resolution that is characteristic of a great number of inflammatory lesions described in joint, lung, gut and kidney (reviewed in Savill, 1997; Savill, 2001). This relationship will be further described (chapter 5). One possible effector cell of apoptosis may be the macrophage, whose arrival on the inflammatory scene can regulate surface expression of FS-7 cell associated cell surface Ag (Fas) ligand and cause the release of soluble Fas ligand, which can induce apoptosis among neighbouring neutrophils (Brown and Savill, 1999). It has been suggested that the process of macrophage phagocytosis of apoptotic neutrophils may initiate a signal in the activated inflammatory macrophage to begin a process of migration from the inflamed local tissue to the lymphatics, the usual fate of inflammatory macrophages (Bellingan, et al, 1996).

Alternatively, uptake of apoptotic cells may stimulate macrophages to release mediators that suppress the inflammatory response, such as TGF-β and IL-10, and also induce reduction of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-12 (Voll et al, 1997). Experimentally, the suppressive effect can be largely inhibited by TGF-β1 neutralizing antibodies and reproduced by application of exogenous TGF-β1, implicating a major role for this anti-inflammatory growth factor in the reduction of these
proinflammatory mediators (McDonald et al, 1999). In vivo and in vitro evidence has shown macrophage secretion of TGF-β1 to suppress pro-inflammatory signalling from Toll-like receptors, and it is thought to be associated with events induced with apoptotic cell contact with macrophages (Byrne and Reen, 2002). Moreover, it has been shown that the intestinal mucosa promotes epithelial restitution after mucosal barrier injury through increased production of bioactive TGF-β1 in epithelial cells (Dignass and Podolsky, 1993) and in subepithelial myofibroblasts (McKaig et al, 1999). In the submucosa, TGF-β1 downregulates apoptosis of fibroblasts and thus may increase matrix deposition (Jelaska and Korn, 2000). Therefore, the reparative properties of TGF-β1 also suggest a link between mechanisms governing resolution of inflammation and engagement of the repair process in damaged tissue. This link will become apparent in the interpretation of results as discussed in Chapter 5.

Therefore, an evident up-regulation of the intestinal immune system and of complex mucosal inflammatory pathways is observed following contact of intestinal tissues with stimulatory bacterial products. This raises the question of whether there is a deficiency in effective down-regulation through the absence of normally suppressive cytokines, such as IL-10, TGF-β, interleukin-4 (IL-4), and IL-1 receptor antagonist (reviewed in MacDermott, 1996). In the intestinal mucosa, the suppression of the active and destructive immunologic and inflammatory events should normally occur following the resolution of a bacterial or viral infection that has been appropriately controlled by the mucosal immune system. However, in IBD, the suppressive events and processes that should turn off the immunologic and inflammatory protective processes appear to be deficient or only partially effective, even upon clearance of the pathogenic agent. Thus
the immune modulation may be dysfunctional. Other possibilities for the persistent inflammation during IBD could be the consequence of persistent stimulation of the normal response as a result of a defect in the mucosal barrier or an epithelial reaction to the indigenous flora (Podolsky et al, 2002a,b; Xavier and Podolsky, 2007; Grbic et al, 2008).

**IBD etiology**

Despite the concerted research efforts during the past few decades, the etiology and pathogenesis of the two major inflammatory bowel diseases, CD and UC, remain rather unclear. Both diseases are thought to result from abnormal and persistent activation of the mucosal immune system driven by the presence of the natural luminal microbiota. Investigators have been making great efforts directed towards the identification of environmental factors, implicating opportunistic commensal bacteria rather than typical exogenous pathogens as drivers of dysregulated immunity and resultant IBD. Recent studies have provided evidence that the risk and severity of IBD are the result of a genetic predisposition that leads to a mucosal immune regulatory cell defect, barrier defects, and susceptibility to environmental triggers, including luminal bacteria and specific antigens (Podolsky et al, 2002a,b; Kucharzik et al, 2006; Strober et al, 2007; Xavier and Podolsky, 2007).
Host Factors

Epithelial Barrier

There is considerable evidence that IBD is characterized by a defective epithelial barrier that allows excessive interaction of the normal mucosal immune system with the intestinal microbiota (reviewed in Strober et al, 2007). Distinct from other epithelia, the intestinal epithelium is composed of a single layer of polarized intestinal epithelial cells, which protects against direct contact of enteric dietary antigens, bacteria or other pathogens with the underlying gut-associated lymphoid tissue (GALT). The presence of mucins, trefoil peptides, the rapid turnover of epithelial cells, and peristalsis, all help to protect against colonization and invasion of the intestinal mucosa by pathogens (Lievin-Le Moal and Servin et al, 2006). In addition, intercellular contacts, such as tight junctions, adherens junctions, and desmosomes, contribute to the maintenance and integrity of this epithelium (Potter et al, 1999). These structures form intimate contacts to restrict the passive flow or diffusion of molecules between epithelial cells. Gassler and coworkers (2001) have demonstrated downregulation of junctional complexes, such as E-cadherin and β-catenin, in human IBD biopsies. Furthermore, the pro-inflammatory cytokines TNF-α, IFN-γ, interleukin 17 (IL-17), and chemokines can induce an increase in epithelial permeability by regulating the tight junctions during mucosal inflammation (reviewed in Kucharzik et al, 2006; reviewed in Xavier and Podolsky, 2007). The significance of increased epithelial permeability across the intestinal epithelial barrier as a fundamental factor in IBD pathogenesis has gained increasing support, particularly as this epithelium represents an interface for genetic and environmental influences (Meddings et al, 1994; Soderholm et al, 1999; Irvine and Marshall et al, 2000; DeMeo et
al, 2002; Berkes et al, 2003; Buhner et al, 2006; reviewed in Goyette et al, 2007). A great number of studies have suggested a genetic contribution to increased intestinal permeability among IBD patients (Meddings, 1997; Wyatt et al, 1997; Ma et al, 1997). Goyette and coworkers (2007) pointed out that some of the IBD loci identified through linkage and association analysis have been suggested to be involved in epithelial integrity, differentiation and transepithelial transport.

The apoptotic rate of the epithelial cells is increased during IBD, and this rapid turnover has been suggested to diminish epithelial barrier function. However, more recent data suggested that rapid apoptosis may allow the loss of ions and water and the entry of small antigens; yet it may not contribute significantly to macromolecule permeability (Bojarski et al, 2001). In addition, TNF-α and interleukin 13 (IL-13) have been identified as cytokines that are important for apoptosis induction and regulation of epithelial barrier function during inflammation (Heller et al, 2005). Zeissig and coworkers (2004) showed that anti-TNF-α treatment during Crohn’s disease repaired epithelial barrier function and down-regulated epithelial apoptosis, whereas tight junctions remained unaffected.

The intestinal epithelial cells of the mucosal barrier participate in the innate immune response of the intestinal mucosa by providing a physical barrier between the exterior environment and the lamina propria, as well as by actively secreting and responding to a variety of cytokines, including IL-8, IL-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), and MCP-1 (Jung et al, 1995), and releasing other immune active molecules, such as defensin and immunoglobulin A (IgA) (Muller et al, 2005). Defective production of these mediators may induce increased destruction of the intestinal mucosa, as observed in IBD. Recent studies have shown that CD patients
exhibit decreased secretion of β-defensins, which are cationic peptides with antibacterial properties known to be produced by colonic epithelial cells as well as by innate immunity cells. It was suggested that in CD patients there is a lack of β-defensin induction and thus a relative deficiency of this antimicrobial molecule (Wehkamp et al, 2003). Later, this abnormality was shown to have a genetic basis, in that a polymorphism in the defensin gene cluster linked with low β-defensin copy number was associated with colonic CD (Fellerman et al, 2006; reviewed in Strober et al, 2007).

Several IBD genetic factors discovered to date affect barrier function, and innate and adaptive immunity (reviewed in Xavier & Podolsky, 2007). A recent study identified a susceptibility locus (5p13.1) for Crohn’s disease on Chromosome 5 (Libioulle et al, 2007). Its associated alleles correlated with quantitative expression of the prostaglandin receptor EP4, which is expressed in intestinal epithelial cells and which regulates epithelial barrier function. Interestingly, Kabashima and coworkers (2002) have shown that EP4 knockout mice are susceptible to mucosal damage induced by dextran sodium sulphate (DSS) colitis, the experimental mucosal irritant used as a model in my studies described herein.

**Innate Immunity**

Mounting evidence has suggested that dysregulation of the innate immune response to the resident microflora or enteric pathogens may lead to chronic inflammation of the intestinal mucosa, leading to IBD (Podolsky et al, 2002a; Goyette et al, 2007). For example, one relevant study has shown that CD patients have a generalized impaired innate immune response as reflected by reduced IL-8 production and neutrophil
accumulation in response to intradermal injection of killed bacteria as well as trauma of the skin or the intestine (Marks et al, 2006). The innate immune system is the first line of defense and provides a rapid response to the resident luminal microflora and to pathogens. The innate immune system has evolved to monitor the resident microflora and to transmit threat signals in response to infection by invasive microorganisms. The immediate activation of innate immunity relies on the recognition by the host of specific pathogen recognition receptors (PRR) of conserved microbial motifs, known as pathogen-associated molecular patterns (PAMPs). PAMPs consist of diverse and distinct molecules from bacteria and viruses, such as lipopolysaccharides, peptidoglycan, flagellin and lipoproteins (Akira et al, 2006).

Knowledge of innate immunity has increased enormously with the discovery of many PRRs, such as the cell surface toll-like receptor (TLR) and cytosolic NOD receptor families. These receptors, which mediate the activation of the mitogen-activated protein kinase (MAPK) pathways for TLRs, and the nuclear factor kB (NF-kB) cascades mainly for NOD, appear to play essential roles in mucosal homeostasis, and alterations apparently contribute to the pathogenesis of IBD. These pathways lead to synthesis of reactive oxygen species (ROS), secretion of chemokine and cytokines that initiate chemotaxis of phagocytotic macrophages, and secretion of antimicrobial proteins by Paneth cells (reviewed in Goyette et al, 2007). Studies have shown that mutations in TLRs, and NODs produce defects in sensing of pathogens and predispose the host to recurrent infections as well as perpetuation of chronic intestinal inflammation (reviewed in Yamamoto-Furusho and Podolsky, 2007).
Studies in experimental models estimate that at least 40 different genes (including known TLR family members) may function in the innate immune response to bacterial invasion (Beutler et al, 2006). The mammalian TLR family consists of 13 members, highly expressed on the surface of monocytes, macrophages, dendritic and epithelial cells. Recognition of microbial components by TLRs triggers activation of signal transduction pathways, inducing dendritic cell maturation, and cytokine production, which then results in development of adaptive immunity (Akira et al, 2006). One of the TLRs most studied due to its association with IBD is TLR4, which is expressed in intestinal epithelium, where it functions as the sensor for lipopolysaccharide (LPS) from Gram-negative bacteria. TLR4 is relevant to IBD, since increased expression of this receptor was observed in the intestinal epithelium of CD and UC patients (Cario and Podolsky, 2000). Polymorphisms of TLR4 have been associated with reduced responsiveness following LPS stimulation (Arbour et al, 2000), and with the development of CD and UC in Caucasian populations (Franchimont et al, 2004; Arnott et al, 2005). Other variants of TLRs associated with IBDs are TLR1 and TLR2, in which polymorphisms were related to UC severity (Pierik et al, 2006).

Another family of PRRs studied for its involvement in IBD pathogenesis is the NOD family. Variations of the NOD2 gene have been definitively associated with increased susceptibility to CD in Western populations (Ogura et al, 2001; Hugot et al, 2001). In peripheral blood mononuclear cells from CD patients, the NOD2 ligand induced decreased TNF-α and IL-1β, but increased IL-8 secretion (van Heel et al, 2005). Furthermore, monocytes isolated from CD patients carrying another variant of NOD2 were reported to exhibit defects in the production of proinflammatory cytokines TNF-α, IL-6 and
IL-8, as well as the anti-inflammatory cytokine IL-10 (Netea et al, 2005). Dendritic cells failed to up-regulate the costimulatory molecules CD80 and CD86, and lack production of cytokines, such as TNF-\(\alpha\), IL-12 and IL-10 (Kramer et al, 2006). Moreover, variants of the NOD1 gene have been shown to be involved in the development of UC and CD in the early onset of the disease (McGovern et al, 2005). However, further investigations need to be completed to prove an association (reviewed in Yamamoto-Furusho & Podolsky, 2006).

Adaptive Immunity

In IBD, the innate immune response plays an essential role in the inductive phase: epithelial barrier defect, production of inflammatory cytokines and defective neutrophil function. This deregulation of innate immunity may be a prerequisite for the excessive activation of adaptive immunity. Therefore, development of IBD seems to involve the exacerbation of the adaptive immune system, characterized by an imbalance between regulatory and effector-cell immune responses to luminal antigens. T-and B-cells are key cells of adaptive immunity. In the intestine and important in IBD patients, B cells produce immunoglobulin A (IgA), including the IgA dimer of secretory IgA, and immunoglobulin G (IgG). T cells are dominated by a Th1, Th17 or Th2 phenotype, and the presence of regulatory T/B cells (reviewed in Danese and Fiocchi, 2006).

Initially, studies showed altered patterns of secretion of IgA and markedly increased levels of IgG antibodies in the systemic as well as mucosal tissues as a consequence of chronic gut inflammation (MacDermott et al, 1986; Scott et al, 2006). However, substantial evidence of tissue injury-inducing autoantibodies in IBD is still missing. Therefore, the focus of IBD immunology investigation shifted to T helper (Th)
cell subsets and the inflammatory mediators that they produce. IBD, in most human cases examined, appears to be associated with a Th1 response (Blumberg et al, 1999). In CD, mucosal T-cells are resistant to apoptosis, and they cycle faster than control cells (Ina et al, 1999; Sturm et al, 2004). Furthermore, intestinal CD4+ T cells produce large amounts of IFN-γ in CD, and they display marked over-expression of the Th1-cell-specific transcription factor (Neurath et al, 2002b), while mucosal macrophages produce increased levels of IL-12 and IL-18 (Monteleone et al, 1997; Pizarro et al, 1999). In addition, IL-23, a heterodimeric cytokine composed of the IL-23A (p19) and the shared IL-12B (p40) subunit (Oppmann et al, 2000), was found to be an essential effector in the development of chronic intestinal inflammation in mice (Hue et al, 2006; Kullberg et al, 2006). Studies have shown that IBD in IL-10- deficient mice could be prevented by a cross with IL-23 -deficient mice, which suggest the involvement of IL-23 in the induction of colitis (Kullberg et al, 2006; Uhlig et al, 2006; Yen et al, 2006). In contrast to CD, in a Th2 colitis model resembling UC, natural killer T cells (NKT-cells) produced increased amounts of IL-13 (Heller et al, 2002), and mucosal T-cells produced more interleukin 5 (IL-5) (Fuss et al, 1996). Moreover, a more prominent increase in eosinophil infiltrate was found in Th2-mediated disease (Iqbal et al, 2002). Furthermore, another CD4 T-cell subset has become the new focus of some studies in IBD. They secrete the cytokine IL-17, which is promoted by IL-23 and suppressed by transcription factors required for Th1 and Th2 cells (Weaver et al, 2007; Kastelein et al, 2007).

Lately, the study of adaptive immunity in IBD has concentrated on possible defects in immunoregulation. Regulatory T cells (Tregs), also known as suppressor T cells, play an important role in the maintenance of intestinal immune homeostasis to enteric
bacterial antigens, and they are crucial for suppressing excessive immune reactivity (Sakaguchi et al, 2005). Activated Tregs down-regulate the immune response by secreting IL-10 and/or TGF-β (Groux et al, 1997; reviewed in Croitoru et al, 2004). They are present in normal intestinal lamina propria (Makita S, et al, 2004). It has been suggested that in IBD, effector T cells might decrease the development of Tregs in the thymus (reviewed in Latinne & Fiasse, 2006). Furthermore, in IBD, there is a substantial increase of Tregs in the blood stream and only a moderate expansion in the inflamed intestine, suggesting a problem with insufficient regulation during active disease (Maul et al, 2005).

Initially, based on the evidence discussed above, it was thought that UC seemed to exhibit an atypical Th2-type-like cytokine profile, and CD, a typical Th1 profile. However, there are no studies that actually demonstrate that Th2 cells in the intestine regulate Th1 cells or vice versa. Thus experimental colitis is not well-explained as an imbalance between Th1 and Th2 subsets. At present, the data are compatible with the concept that excessive responses of either the CD4⁺ Th1 or Th2 effector subsets are detrimental and can result in IBD. Therefore, a more complex and significant overlap between the two major forms of IBD has been suggested (reviewed in Elson et al, 2005; Danese and Fiocchi, 2006; Shi et al, 2006; Xavier and Podolsky, 2007; Goyette et al, 2007).

**External environmental factors**

Diet has been suggested to play an important role among various environmental factors in IBD. Fast-food containing large amounts of fat, and/or refined sugar, has been
found to accelerate the development of CD (Sonnenberg et al, 1988; Sakamoto et al, 2005). On the other hand, studies have shown that the administration of omega-3 fatty acids has a positive effect on IBD (Mahmud et al, 2001; Cashman et al, 2003; Yuceyar et al, 1999).

Some studies have reported an association between smoking and IBD in Chinese patients. Patients with a history of smoking were more susceptible to CD (Leong et al, 2004), while no relationship between smoking and severity of UC was reported in another large population of Chinese patients (Jiang et al, 2002). Another risk factor is the consumption of NSAIDs, which has been shown to induce a more rapid and severe form of colonic inflammation in IL-10 knockout mice that spontaneously develop colitis (Berg et al, 2002). In contrast, stress is more likely to modulate disease manifestations rather than being an initiating factor of IBD (Collins, 2001). Exacerbations of clinical disease activity appear to be associated with sustained but not short-term stress (Levenstein et al, 2000).

The natural intestinal microbiota in IBD

It has been suggested that bacteria are essential for IBD, since certain strains of mice which spontaneously develop colitis, when germfree, do not develop intestinal inflammation (Madsen et al, 1999). Indeed, inflammation appears rapidly when germ free animals are reconstituted with bacteria that are considered normal constituents of the luminal microbiota (Elson et al, 2005; Onderdonk et al, 1977). Therefore, the evidence acquired to date indicates that the balance between microbes, especially the commensal
microflora, and the host immune responses at the mucosal barrier is a critical factor in the initiation and pathogenesis of IBD.

In support of this hypothesis, the therapeutic benefits of antibiotic treatment have been observed in subsets of IBD patients, who experienced sustained IBD remission (Prantera et al, 1996; Rutgeerts et al, 1995). This suggests that altering the pattern of bacterial colonization may have an impact on intestinal disease. Indeed, treatment with probiotic combinations ameliorate IBD (Sutherland et al, 1991; Kanauchi et al, 2002; Gionchetti et al, 2003; McCarthy et al, 2003; Kanauchi et al, 2003; Di Giacinto et al, 2005). In addition, it has been shown that various commensal bacterial species selectively induce disease in hosts with different genetic backgrounds, and they may cause different outcomes of disease in a single genetically susceptible host (Kim et al, 2005). In contrast, studies in animal models show that there are differences in the colon microbiota between adult normal mice and colitis-prone C3H mice (Elson et al, 2005). Yet, these changes are possibly a consequence of abnormal mucosal immune responses, since it has been shown that the ability of mice to mount innate responses affects the composition of the gut commensal microflora (Niewenhuis et al, 2009). These investigations provide convincing evidence that the nature of the host defenses and their modulation, rather than toxigenic properties of a luminal bacterial species, per se, may determine the occurrence of IBD.

**IBD treatment**

A specific treatment for human IBD, based on its cause, is not available. Until more is understood about the etiology of IBD, non-specific treatments with various
agents are the standard defense against CD and UC. Therefore, patients are not only burdened by the symptoms of IBDs, but they are also troubled by the side-effects of the therapeutics. The “classical” treatment for IBD consists of 5-aminosalicylic acid (5-ASA), steroids and other immunosuppressants. Unfortunately, due to failure of conventional medical therapy, about 33% to 50% of patients with fulminant ulcerative colitis who are admitted to the hospital have their colon removed (Travis et al, 1996). Therefore, new treatment options for IBD have been explored, such as biological and probiotic therapeutics. These prominent therapies will be discussed in the following paragraphs.

**Biological therapy**

One of the best strategies to effectively down-regulate the exacerbated immune response may be to interfere with multiple stages of the inflammatory cascade (reviewed in Kho et al, 2001). Biological therapy, which refers to the use of medication that is tailored to specifically target an immune or genetic mediator of disease (Staren et al, 1989), has been used for the treatment of IBD, being one of the major achievements since the introduction of steroids. Theoretically, it represents a more specific management of this disease with fewer side effects. Drugs such as infliximab, CDP571, adalimumab, and certolizumab have been important treatment advancements because they allow the direct targeting of the inflammatory cytokine TNF-α. Among these biologics, only infliximab has emerged as a therapeutic option for CD, and it has also shown to be effective in some patients with UC.
The number of TNF-α-producing cells is greatly increased in the lamina propria in the bowel of patients with Crohn’s disease (Reinecker et al, 1993; Breese et al, 1994) and increased concentrations of TNF-α have been found in the stools of children with Crohn’s disease (Nicholls et al, 1993). Infliximab (Remicade, Centocor) is an intravenously administered chimeric monoclonal immunoglobulin (Ig) G1 antibody to TNF-α. Studies have shown T cell apoptosis after treatment with infliximab, with increased effect in activated T cells (Lugering et al, 2001; ten Hove et al, 2002). Infliximab reduces permeability of the intestinal barrier, restoring the epithelial barrier function (Gibson, 2004). Also, it deactivates the endothelium by downregulating the expression of vascular cell adhesion molecule-1 (VCAM-1) and CD40 expression in the intestinal mucosal endothelium (Danese et al, 2006), and it induces intestinal fibroblast motility, which can lead to intestinal wound healing (Di Sabatino et al, 2007; Danese, 2008).

**Helminthic therapy**

A recent hypothesis suggests that some IBD cases are caused by an overactive immune system attacking various tissues of the digestive tract because of the lack of traditional targets such as parasites and worms. The inverse relation between the frequency of worm colonization and the prevalence of IBD was first hypothesized (Elliott et al, 2000) and then supported by an epidemiological study (Weinstock et al, 2004). Recently, a few studies in animals using dextran sulphate sodium (DSS), trinitrobenzene sulfonate (TNBS), and dinitrobenzenesulfonic acid (DNBS) colitis models have shown that different helminth parasites (nematode, cestode, and trematode) can ameliorate colitis (Reardon et al, 2001; Khan et al, 2002; Elliot et al, 2003, Elliot et al, 2004).
Moreover, clinical studies have shown protective effects of helminthic therapy against UC and CD when patients were treated with *Trichuris suis*, and *Necator americanus* (Summers et al, 2005a; Summers et al, 2005b; Croese et al 2006). The importance of regulatory pathways, such as regulatory T-cells, by which helminths induce such protection have been described. However, the complex pathways that helminths activate to regulate the host's immune system need further investigation (Ruyssers et al 2008).

**Animal models used in IBD studies**

Experimental models of IBD are valuable tools for answering questions that are difficult to address in humans. They are used for studying many aspects of disease, especially the mechanisms that mediate the pathogenesis in the early phases of colitis, since IBD patients often come to clinics only after their symptoms are established. Animal models are also important in investigations of the effect of new therapeutic strategies. One of their great advantages is that the environmental conditions and genetics can be either controlled or defined. The value of a well defined model is that it may bring clarity to the complex processes and mechanisms that can result in IBD. However, these models do not necessarily represent the complicated nature of human disease accurately, and they cannot substitute for studies of patient material. Therefore, there is a need for caution in interpreting the value of the data from experimental models.

The availability of murine immunological reagents and the fact that the mouse genome has been fully characterized optimizes the utility of murine models. Pizarro and coworkers (2000) indicated that an ideal mouse model to study IBD pathogenesis should resemble the human disease and develop spontaneous intestinal inflammation. Moreover,
in a review paper, Jurjus and coworkers (2004) postulated that an appropriate IBD animal model should exhibit certain characteristics: the intestine should reveal morphological alterations, inflammation, symptoms and signs, pathophysiology, and a disease course similar to human IBD. They also indicated that the animal selected should have a well-defined genetic background.

Most of the experimental models of colitis that have been described are based on a chemical irritant, immune cell transfer, gene targeting (including gene knockout and transgenic), and models in which the mucosal inflammation develops spontaneously. In this section, I describe these categories, with particular emphasis on the Dextran Sulphate Sodium (DSS)-induced colitis model, which is the chemical model used to test the hypothesis of my study.

**Spontaneous mouse models of colitis**

As mentioned above, it is thought that IBD affects individuals who have a genetic predisposition, under the influence of certain environmental trigger factors. Therefore, spontaneous colitis in animal models could present some advantages over inducible models for showing genetic susceptibility factors of mucosal inflammation. Although the occurrence of spontaneous colitis in mice is uncommon, there are some instances in which colitis has occurred at high frequency in a strain of mice, such as in C3H/HeJBir mice and SAMP1/Yit mice (reviewed in Elson and Weaver, 2005).

C3H/HeJBir is a substrain of the C3H/HeJ mouse that was generated more than 15 years ago at the Jackson Laboratory, and that is highly susceptible to the development of colitis
(Sundberg et al, 1994). These mice reproducibly develop a spontaneous pathogen-independent colitis at about 3 or 4 weeks of age, with acute and chronic lesions and ulcerations mainly in the cecum and the mucosa of right side of the proximal colon (Sundberg et al, 1994). They present a toll-like receptor 4 mutation, which renders them unresponsive to bacterial endotoxin (LPS), but they do recognize a select number of enteric bacterial antigens (Brandwein et al, 1997). Increased secretory IgA, high titer serum antibodies to commensal bacterial antigens, and increased T cell responses to orally delivered antigen have been found in C3H/HeJBir mice (McCabe et al, 1994). Notably, C3H/HeJBir mice exhibit defects in innate immunity that provokes increased T-cell responses to bacterial antigens (Elson et al, 2005).

Another model of spontaneous intestinal inflammation is the SAMP1/Yit mouse strain and the SAMP1/YitFc substrain. Unlike most of the IBD models described in this review, the intestinal inflammation in SAMP1/Yit mice develops in the small intestine (Matsumoto et al, 1998); the absence of colonic involvement is characteristic (Kosiewicz et al, 2001), which is similar to human CD. Evidently, both Th1 and Th2 pathways mediate disease development in SAMP1/Yit mice (Olson et al, 2004).

**Adoptive transfer mouse models**

Intestinal inflammation is developed in adoptive transfer models by passage of certain cell types, such as T cells or bone marrow precursors, to immunocompromised mice. Studies in these models have shown strong evidence that Th1 polarization plays a key role in CD pathogenesis (Powrie, 1995). However, in a review paper, Pizarro and coworkers (2003) pointed out that the great immune abnormalities in recipient mice
probably make these models inappropriate for studying the innate factors that cause
human colitis.

One of the most studied adoptive transfer models consists of transferring CD4\(^+\) T
cells, expressing high levels of the surface molecule CD45RB, from wild-type donor
mice to immunodeficient severe combined immunodeficiency (SCID) or recombination
activating gene (RAG) deficient recipient mice. This adoptive transfer results in disease
manifested by chronic non-bloody diarrhea with intestinal inflammation, starting 5–8
weeks after cell transfer (Leach et al, 1996; De Winter et al, 1999; Powrie et al, 1993;
Bregenholt et al, 1999). Histopathologic changes in the recipients, which appear very
similar to those of other models of colitis, are limited mainly to the colon, which is
markedly thickened due to hyperplasia (Elson et al., 1995). Another model that has been
very useful in IBD studies is characterized by the adoptive transfer of a heat shock
protein 60 (hsp60)-specific CD8\(^+\) T-lymphocyte clone, pre-activated by bacterial hsp60,
into T cell receptor (TCR)\(^{-/-}\) or SCID mice (Steinhoff et al., 1999). This transfer results
in severe, generally lethal intestinal lesions, predominantly in the small intestine. In
contrast to the findings obtained in many other models, intestinal inflammation in this
model does not depend on the presence of the resident bacterial flora (Steinhoff et al,
1999).

**Genetically engineered mouse models**

Genetic models consist of transgenic and knockout models, which are
characterized by a particular genetic disturbance produced by either gene targeting or the
introduction of a transfected gene. The majority of the genetic mouse models in this
group are gene knockouts, which are mainly represented by the IL-2 (Bush et al, 1998), T-cell receptor (TCR)α/β (Mombaerts et al, 1993), IL-10 (Kuhn et al, 1993), TGF beta (Kulkarni et al, 1995), TNF-α (Kontoyiannis et al, 1999) and Gi2-α (Rudolph et al, 1995) knockout models. Also, there are a few transgenic mouse models, which are mainly represented by the E-cadherin (Hermiston et al, 1995), STAT 4 (Wirtz et al, 1999), interleukin 7 (IL-7) (Watanabe et al, 1998) transgenic mice. Many of these models include molecules involved in cytokine functions, which could include targeting cytokines, cytokine receptor (TCR), and the antigen-presenting complex. Others among these models affect the intestinal epithelial barrier function, which can be targeted in various ways. Genetic models allow for the determination of how and why particular immunologic defects lead to mucosal inflammation (Blumberg et al, 1999). Despite all the benefits that these models provide to our understanding of the pathogenesis of IBD, it is unlikely that the induced genetic mutations correspond to the underlying defect in human IBD, thereby limiting the utility of these models for the comprehension of the etiology of UC and CD (Pizarro et al, 2003).

**Chemically-induced mouse models**

This group of animal models is characterized by colitis that is induced by chemical irritation following the administration of diverse agents: sulfated polysaccharides, such as carrageenan (Marcus et al, 1989), oral DSS (Okayasu et al, 1990); hapten reagents diluted in ethanol, such as intrarectal insertion of trinitrobenzene sulfonate (TNBS) (Morris et al, 1989), intrarectal insertion of oxazolone (Boirivant et al, 1998); intrarectal insertion of diluted acetic acid (MacPherson et al, 1978); and
intravenous injection of immune complexes followed by chemical irritation of the colon. Each of these models involves a chemical stress or other injury to elicit disease. Some are limited to acute tissue injury; in others, the tissue injury is part of a more complex immunologic reaction. In some important immunological and histopathological aspects, they resemble IBDs in humans. All of these models exhibit disruption of the epithelial barrier, consequently increasing the exposure of intestinal microflora to the intestinal immune system. In addition, these chemicals induce lesions with similar histological findings, which suggest that colitis is perhaps a nonspecific and conventional injury in response to a chemical insult (Rivera-Nieves et al, 2008).

Chemically induced models of intestinal inflammation are the animal models most commonly used for IBD. Different from other categories, they lead to the rapid onset of inflammation, and the procedure is relatively simple. By using these systems, important information has been gathered regarding the pathogenic role of specific cytokines in experimental colitis (Neurath et al, 1995). They are valuable for studying biochemical pathways of inflammation or for performing antigen-specific studies, such as in the case of hapten-induced gut inflammation (e.g. TNBS). In addition, they are relatively inexpensive for providing proof of concept for therapeutic interventions (Pizarro et al, 2003).

Although they are important tools to study the pathogenesis of IBD, like all other models, chemically induced IBD models do present limitations. They are definitely different from human IBD, including the initiating events as well as the clinical course of disease (Yamada et al, 1992). In addition, the inflammatory response in these models resolves spontaneously upon removal of the injurious agent. Consequently, these models
do not accurately reproduce the specific chronic pathogenic mechanisms that mediate and sustain disease states in human UC or colonic CD.

**DSS-induced Colitis Model**

Feeding mice (Okayasu et al, 1990; Cooper et al, 1993), rats (Tamaru et al, 1993), hamsters (Yamada et al, 1992) or guinea-pigs (Iwanaga et al, 1994) for several days with 30-50 kDa dextran sulfate sodium (DSS), a polymer of sulfated glucose, at 3-10% dissolved in the drinking water, induces an acute colitis. DSS-induced early lesions occur mainly in the distal colon and over lymphoid aggregates. The inflammation is characterized by bloody diarrhea, weight loss, colon shortening, neutrophil infiltration, and histological lesions characterized by epithelial changes, fibrosis, crypt loss, goblet cell hypoplasia, and focal ulceration (Okayasu et al, 1990; Mahler et al, 1998; Melgar et al, 2005; Wirtz et al, 2007).

It is generally believed that DSS is directly toxic to gut epithelial cells of the basal crypts. The DSS protocol is the most frequently used model that has acute disruption of the mucosal barrier (Cooper et al, 1993; Kitajima et al, 1999). Indeed, DSS inhibits proliferation of mouse epithelial cells *in vitro* (Dieleman et al, 1994), and it has been shown to be suitable to study epithelial barrier immune defence and repair mechanisms in murine colons (Williams et al, 2001). Studies using TLR4−/− and MyD88−/− mice suggest that TLR signalling is necessary to limit bacterial translocation after DSS- induced intestinal epithelial damage, suggesting that TLR signalling is crucial for the preservation of the epithelial barrier (Fukata et al, 2005).
The pathogenesis of colitis in the DSS model differs from that in other models in which IBD is brought about by cytokine imbalances induced by genetic disruption of specific cytokine genes. Notably, a requirement for functional T and B lymphocytes in the DSS model has been excluded (Dieleman et al, 1994; Axelsson et al, 1996; Mahler et al, 1998). Indeed, DSS induces an acute inflammatory condition that is more dependent on innate immune responses, as it develops even in the absence of T, B, and NK cells (Axelsson et al, 1996). Moreover, the fact that T- and B-cell-deficient SCID or recombination activating gene 1 (RAG1)^−/− mice also develop severe colitis suggests that the adaptive immune system does not play a major part in pathogenesis, at least in the acute phase, in this model (Dieleman et al, 1994). Yet, in mice with an intact immune system, there is eventual activation of T-cell responses, which is superimposed on the primary innate responses induced by the chemical agent. Furthermore, the absence of a T or B lymphocyte requirement in the DSS model may, in part, be explained by the fact that DSS itself is a potent macrophage activator (Okayasu et al, 1990). Within a day of administration, DSS can be taken up by macrophages that remain in the lamina propria or later migrate to mesenteric lymph nodes, liver, and other organs (Okayasu et al 1990; Kitajima et al, 1999). Consequently, the acute DSS colitis model is mainly useful to study the involvement of innate immune mechanisms of colitis.

Among susceptible strains, such as the Swiss-Webster mouse, the administration of DSS for 3-5 cycles (7 days DSS, 14 days water alone) results in chronic lesions with infiltrating macrophages, CD4+ T lymphocyte aggregates in the lamina propria and serosa, a patchy distribution of inflammation, and fissuring ulcers (Okayasu et al, 1990; Cooper et al, 1993; Mahler et al, 1998). Later phases of the disease are associated with
increased levels of proinflammatory cytokines (IL-2, IL-4, IL-6), leukotriene B4 and thromboxane, which indicates the involvement of the adaptive immune system. Moreover, increased levels of IFNγ and IL-4 were found in the mucosa during chronic colitis (Dieleman et al, 1998). The observation that treatment with cyclosporine A had a beneficial therapeutic effect on the chronicity of colitis indicates the involvement of T cells in DSS- induced chronic colitis (Murthy et al, 1993). In a recent study, that used DSS to induce acute colitis (3% DSS for 7 days) and chronic colitis, cytokine profiles were analyzed. Increased TNF-α, IL6, and IL-17 profiles were found in acute colitis; in the chronic state, a predominant Th2-mediated inflammatory response (increase in IL-4 and IL-10 and concomitant decrease in TNF-α, IL6, and IL-17) was found (Alex et al, 2009).

It is still not clear whether intestinal bacteria contribute directly to tissue injury in the DSS colitis model. There are studies that have suggested that luminal bacteria may be a factor in the pathogenesis of the DSS-induced lesions, since colonic cell counts of Bacteroides spp., especially B. distasonis, are increased in acute and chronic phases of inflammation (Okayasu et al, 1990). In addition, antibiotic therapy with either metronidazole, vancomycin, or imipenem prevents, or can treat, DSS colitis, and treatment with probiotic organisms can ameliorate DSS colitis (Yamada et al, 1992; Verdu et al, 2000; Rath et al, 2001; Setoyama et al, 2003; Rachmilewitz et al, 2004). In contrast, bacterial LPS does not seem to contribute to the pathogenesis of DSS colitis, since the LPS-resistant mouse strains C3H/HeJ and C3H/HeJBir remain very susceptible to DSS-induced colitis (Mahler et al, 1998). Furthermore, Kitajima (2001) has shown that
germ-free mice develop DSS colitis to the same, or even more severe degree, as normal mice.

Patients with UC have been reported to have an increased risk for the development of colon cancer (Jess et al, 2006). Chronic DSS-induced colitis, if combined with a single initial dose of the genotoxic colon carcinogen azoxymethane (AOM), results in colorectal tumors predominantly in inflamed regions of the colon (Okayasu, 1996; Tanaka et al, 2003). Moreover, some other studies have shown that prolonged low-dose feeding of DSS has resulted in colitis, dysplasia, colonic adenomas, adenocarcinomas, and papillomas in hamsters (Yamada et al, 1992) and rats (Hirono et al, 1981; Tamaru et al, 1993). As colonic inflammation is thought to play a key role in IBD-related colorectal cancer, experimental DSS colitis might also be a valuable model for studying the molecular mechanisms linking colitis to colorectal carcinogenesis.

The DSS model has a number of more direct advantages, and it is a technically simple method to induce colitis in most mouse strains. Differential susceptibility to DSS has been reported for several mouse strains; nevertheless this chemical induces some degree of colitis in all mice (Mahler et al, 1998). The DSS model exhibits many signs that are similar to those seen in human UC, and by using this model, it is possible to induce both the acute and the chronic phase of UC (Okayasu et al, 1990; Cooper et al, 1993). The lesion, the time course, and the severity of colitis are rather uniform and reproducible (Murthy et al, 1993). The clinical and histologic severity can be quantified, making this model very useful and reliable (Arai et al, 1998). Consequently, DSS-induced colitis has been recognized to be very valuable as a test for potential therapeutic agents, and it has
been considered a sensitive screening system (Elson and Weaver, 2005). Indeed, the DSS colitis model has provided utility for testing epithelial repair agents, biologicals (e.g., probiotics using Clostridium butyricum and Lactobacillus casei) (Okamoto et al, 2000; Herias et al, 2005), and cytokine inhibitors (e.g., to TNF-α, IL-18 and IL-1) (Kojouharoff et al, 1997; Arai et al, 1998; Sivakumar et al, 2002). Furthermore, due to its lesion reproducibility, it allows identification and mapping of genes important for susceptibility to colitis. Indeed, the location of a number of candidate genes has been identified through quantitative trait locus mapping (Mahler et al, 1999). The major limitation of this model is mainly related to the fact that it represents a nonspecific injury model that requires neither T cells nor B cells. Therefore, it is not well suited to address complex immunologic or therapeutic issues involving the acquired immune system (Elson and Weaver, 2005).

For my study, experimental colitis was induced in mice, using the DSS colitis model, for testing a hypothesis regarding osteopontin’s function as a modulator of innate immunity. In this investigation, I used clinical, histological, haematological and biochemical methods to determine the effect of endogenous OPN in the pathogenesis of colitis and the potential impact of administering exogenous OPN to attenuate experimental colitis. The next section of chapter 1 and chapter 2 constitute a review of significant relevant literature that focuses on OPN.

**Osteopontin (OPN)**

The multifunctional role of OPN in inflammatory diseases is well established (reviewed in Sodek et al, 2000; Denhardt et al, 2001; Sodek et al, 2006; Singh et al, 2007;
Scatena et al, 2007; Ramaiah et al, 2008). In inflammatory situations, OPN is thought to promote both pro- and anti-inflammatory responses, which can be either beneficial or harmful depending on what other stimuli the cells are receiving (reviewed in Wang and Denhardt, 2008). Chapter 2 of this thesis is a review paper that I co-authored with my supervisors, “Osteopontin and Mucosal Protection”, on OPN structure and details about its functions during stages of inflammation. In the publication, data relevant to my thesis are discussed, covering studies on the relationship of OPN to innate immunity and mucosal protection during inflammation. To avoid redundancy, this Introduction concentrates on various molecular forms of OPN and their extra- or intra-cellular compartmentalization, in light of OPN’s multifunctional roles in tissue remodelling and inflammatory responses.

**Intracellular form of OPN (i-OPN)**

A recent study reported that i-OPN and secreted OPN (OPN-s) represent alternative translational products of a single full-length *OPN* mRNA (Shinohara et al, 2008). It showed that i-OPN, the structure of which is the consequence of translation initiation downstream of the usual start site, appears as a shortened protein that lacks the N-terminal signal sequence and localizes mainly to the cytoplasm. Furthermore, there is much evidence for the existence of an intracellular form of OPN, localized to the perimembranous region. It has been shown to co-localize primarily with the cytoplasmic domain of CD44 and ezrin-radixin-moesin (ERM) proteins that connect the cortical cytoskeleton with the plasma membrane, in migrating embryonic fibroblasts, activated macrophages, and metastatic breast cancer cells (Zohar et al, 1998; Zohar 2000; reviewed
in Sodek et al, 2000). OPN associates with these proteins only in the presence of CD44 (Zohar et al, 2000). As a consequence, i-OPN has been implicated in a growing number of cellular processes, including migration, fusion, survival and motility (Zohar et al, 2000; Suzuki et al, 2002; Zhu et al, 2004; Junaid et al, 2007).

A specific role of i-OPN in cell migration was first reported by Zohar (1997). Subsequent to this study, other reports showed its involvement in the migration of embryonic fibroblasts, activated macrophages, and metastatic cells (Zohar et al, 2000; Zohar et al, 2004). Impaired chemotaxis of OPN−/− macrophages (Zhu et al, 2004) and osteoclasts (Suzuki et al, 2002) has been associated with reduced cell surface expression of CD44, and studies have shown that OPN expression is required for the recruitment of CD44 to the cell surface of osteoclasts (Chellaiah et al, 2003b). In macrophages, as well as in osteoclasts and fibroblasts, CD44 co-localizes with OPN inside lamellipodia and cell processes (Zhu et al, 2004; Suzuki et al, 2002; Zohar et al, 2000), which may reflect its contribution to actin polymerization at the leading edge during migration, consistent with its association with the ERM complex that couples cell surface adhesion molecules with actin filaments (Zohar et al, 2000). Furthermore, a mutual dependence of i-OPN and CD44 cell surface expression has been demonstrated in both macrophages and osteoclasts (Suzuki et al, 2002; Zhu et al, 2004). These interactions may explain how i-OPN mediates cell migration.

Notably, there is also evidence that suggests that i-OPN has other cellular functions, particularly in relation to cell proliferation (Elgavish et al, 1998; Tuck et al, 1997; Weintraub et al, 2000). Recently, a study implicated i-OPN as a participant in the process of cell division (Junaid et al, 2007). Furthermore, recent evidence has revealed
that i-OPN expressed by dendritic cells regulates the expression of pro-inflammatory cytokines and the differentiation of T helper-cell lineages (reviewed in Cantor and Shinohara, 2009). Shinohara (2006) showed that i-OPN induces IFN-α expression in plasmacytoid dendritic cells by selectively coupling TLR9 signaling to expression of IFN-α, whereas expression of i-OPN in conventional dendritic cells promotes differentiation of IL-17-producing T helper cells (Th17 cells) (Shinohara et al, 2008). This indicates that intracellular and secreted OPN may differentially regulate signaling pathways in various cell lineages to influence the type and intensity of immune responses (reviewed in Wang & Denhardt, 2008). When interpreting my data relating to OPN’s potential function as an immune modulator, I suggest below that the expression of i-OPN by macrophages probably determines the degree to which OPN-s, or exogenously administered, OPN attenuates experimental colitis (see discussion, chapters 5 and 6).

**Post-translational modifications of OPN**

OPN exists in a number of distinct isoforms that differ in the degree of post-translational modifications (PTMs), such as phosphorylation, glycosylation, sulfation, and proteolytic processing, which have substantial impacts on the structure and biological functions of the protein (reviewed in Kazanecki et al, 2007). OPN phosphorylation has been well characterized since it has significant functional implications.

The degree of OPN phosphorylation varies, depending on the tissue source, cell type, and the differentiation stage. For example, bovine milk OPN contains 27 phosphoserine and 1 phosphothreonine residues (Sørensen et al, 1995). Greater
phosphorylation was reported for the human milk protein, which contains a total of 34 phosphoserines and two phosphothreonines (Christensen et al, 2005). In contrast, rat bone OPN has an average of 12 phosphoserines and 1 phosphothreonine (Prince et al, 1987; Keykhosravani et al, 2005). It has been suggested that the reason that milk OPN is more highly phosphorylated than bone OPN may be due to the harsh methods required for purification of OPN from bone, which could result in loss of some phosphates (Kazanecki et al, 2007). Moreover, different forms of OPN that vary in their phosphorylation have been reported in transformed osteogenic cells (Kasugai et al, 1991; Safran et al, 1998). Furthermore, differentiating rat osteoblasts produce two forms of OPN: a 55-kDa form that contains little phosphorylation and a highly phosphorylated 44-kDa form (Sodek et al, 1995). The extent of phosphorylation of OPN in osteoblast and epidermal cells is responsive to hormonal influences, such as calcitriol (the active form of vitamin D) (Safran et al, 1998; Chang et al, 1991).

Diversity in OPN phosphorylation may explain the multitude of biological functions of OPN in different tissues and body fluids. However, the exact effect of phosphates on this protein is still unknown. It has been shown that dephosphorylated OPN loses the ability to inhibit hydroxyapatite formation (Boskey et al, 1993; Hunter et al, 1994). Moreover, the highly phosphorylated bovine milk OPN promotes hydroxyapatite formation and growth, whereas the bovine bone OPN with much less phosphorylation inhibits formation (Gericke et al, 2005). Other studies have shown that phosphorylation is critical for OPN inhibition of calcium oxalate crystallization in urine (Hoyer et al, 2001) and calcification of vascular smooth muscle cells (Jono et al, 2000). Furthermore, Christensen (2007) showed that OPN produced by murine transformed
fibroblasts and differentiating osteoblasts demonstrated different phosphorylation patterns, which translated into significant functional differences in cellular adhesion. The fibroblasts cell, with an OPN that has fewer phosphates groups, had much greater adhesive properties. Also, phosphorylation of OPN has been shown to promote migration of cancer cells (Al-Shami et al, 2005).

There are some studies that addressed the role of phosphorylation of OPN in immune cells. In indicated previously, OPN can influence the production of cytokines by interaction with surface receptors on immune cells. Ashkar (2000) reported that phosphorylation in the N-terminal domain of OPN is required for RGD-β3-integrin recognition or binding, and subsequent induction of interleukin-12 expression in mouse peritoneal macrophages. Likewise, phosphorylation is required for the OPN-RGD mediated spreading and activation of macrophages (Weber et al, 2002).

Osteopontin with other types of PTMs have been reported in most tissues (Sodek et al, 2000). Three glycosylation sites for O-linked oligosaccharides have been identified in bovine milk OPN (Sorensen et al, 1995). However, little is known about their specific functions (reviewed Sodek et al, 2000). Sulphation has been shown to occur predominantly in the highly phosphorylated form of OPN (Nagata et al, 1989, reviewed in Sodek et al, 2000). However, its functional implication is unclear.

It is well known that OPN peptides are found in vivo. Thrombin and matrix-metalloproteinases (MMPs) will degrade OPN causing the release of peptides that are active in a variety of functions (reviewed in Scatena et al, 2007). The biologically active thrombin cleavage site, which lies within six amino acids of the RGD sequence,
modulates integrin binding and protein function (Senger et al, 1985; Senger et al, 1996; O’Reagan et al, 1999). Most of the recognized biological activity of OPN resides in the N-terminal thrombin cleaved fragment (reviewed in O’Regan, 2003). For example, this fragment of OPN induces chemotactic and cytokine responses of macrophages through the CD44 receptor (Ashkar et al, 2000; Weber et al, 1996). Furthermore, it has been suggested that the thrombin fragmentation of porcine OPN, which yields two fragments (20 kDa, 23 kDa), might be important in bone formation (Zhang et al, 1990). As per MMPs, three cleavage sites have been identified and one of them, Gly$^{166}$ Leu$^{167}$, lies directly upstream of the thrombin cleavage domain (Agnihotri et al, 2001). OPN fragments of 32 and 40 kDa, which are consistent with in vivo MMP-3/MMP-7 cleavage at Gly$^{166}$ Leu$^{167}$, have been identified (Agnihotri et al, 2001). Yet, further studies are required to define the biological significance of MMPs processing of OPN.
Statement of the Problem, General Hypothesis and Objective

OPN expression is frequently upregulated in response to various pathological stressors (reviewed in Wang and Denhardt, 2008). Gassler and coworkers (2002) showed that OPN is constitutively expressed in normal intestines, which suggested that OPN may be involved in intestinal immune homeostasis. Indeed, our preliminary data showed increased mucosal expression of OPN during experimental colitis. OPN contributes significantly to the progression and sequelae of some experimental inflammatory diseases: arthritis, kidney and lung fibrosis, and multiple sclerosis (Miyazaki et al., 1995; Noiri et al., 1999; Chabas et al., 2001; Yumoto et al., 2002). Yet, since OPN is expressed by epithelial and mucosal cells as well as immune cells (Denhardt et al, 2001) and may function as a cytokine by modulating cell-mediated immunity (Ashkar et al, 2000), it is conceivable that OPN is important both for development of inflammatory bowel diseases (IBD) and protection of the intestines from pathogens. Therefore, I hypothesized that OPN has significant protective functions during colitis, where an efficient mucosal immune response and an intact intestinal epithelial barrier are essential.

The Specific Objective

To determine the functional and clinical significance of OPN in DSS-induced colitis.
CHAPTER 2: Osteopontin and Mucosal Protection

**ABSTRACT**

Protection of mucosal tissues of the oral cavity, intestines, respiratory tract, and urogenital tract from the constant challenge of pathogens is achieved by the combined barrier function of the lining epithelia and specialized immune cells. Recent studies have indicated that osteopontin (OPN) has a pivotal role in the development of immune responses and in the tissue destruction and the subsequent repair processes associated with inflammatory diseases. While expression of OPN is increased in immune cells— including neutrophils, macrophages, T and B-lymphocytes— and in epithelial, endothelial, and fibroblastic cells of inflamed tissues, deciphering the specific functions of OPN has been difficult. In part, this is due to the broad range of biological activities of OPN that are mediated by multiple receptors which recognize several signaling motifs whose activities are influenced by post-translational modifications and proteolytic processing of OPN. Understanding the role of OPN in mucosal inflammation is further complicated by its contributions to the barrier function of the lining epithelia and the complexity of the specialized mucosal immune system. In an attempt to provide some insights into the involvement of OPN in mucosal diseases, this review summarizes current knowledge of the biological activities of OPN involved in the development of inflammatory responses and in wound healing, and indicates how these activities may affect the protection of mucosal tissues.
INTRODUCTION

Osteopontin (OPN) is a phosphorylated glycoprotein that is expressed by a broad range of tissues and cells (Sodek et al., 2000). Although originally characterized as a bone matrix protein (Prince and Butler, 1987), T-lymphocyte activation protein (Eta-1) (Patarca et al., 1993), and cell transformation-associated protein (Craig et al., 1988), OPN functions as a matricellular protein with diverse biological activities mediated by multiple cell-surface receptors (Giachelli and Steitz, 2000). Interest in OPN has focused on its role as an inflammatory cytokine in response to recent studies showing that OPN is up-regulated in inflammatory diseases and is required for the development of cellular immunity (Ashkar et al., 2000; O’Regan, 2003) and wound healing (Liaw et al., 1998; Rittling et al., 1998). In the absence of OPN, the development of many inflammatory diseases is attenuated (Miyazaki et al., 1995; Noiri et al., 1999; Chabas et al., 2001; O’Regan et al., 2001; Jansson et al., 2002; Yumoto et al., 2002). Indeed, OPN expression seems to be up-regulated in almost every wounded organ, including: brain, liver, gastrointestinal tract, lung, bone, cardiac tissue, joints, and kidney and various tumors. Moreover, OPN expression during inflammation is not limited to specific cell lineages but involves many different cells, including epithelial, mesenchymal, as well as immune cells, in the inflamed tissues (Fig. 2.1). The increased expression of OPN is associated with increased cell mobilization, survival, and activity and is reflected in elevated concentrations of OPN in tissue fluids and plasma, which has potential diagnostic and prognostic value for cancer progression and tumor burden (Tuck et al., 2003), as well as for inflammatory disease progression in the joints, cardiac and nervous systems, and in
the intestines (Reinholt et al., 1990; Gassler et al., 2002; Ohshima et al., 2002a,b; Koguchi et al., 2003; Tamura et al., 2003; Vogt et al., 2003).

Recent reports of increased OPN expression, as well as increased plasma levels, in the epithelial and submucosal layers of the intestines in inflammatory bowel diseases (Qu and Dvorak, 1997; Gassler et al., 2002) indicate that OPN has important functions in protecting mucosal surfaces (Fig. 1). However, there have been few studies of OPN in the normal or diseased mucosal tissues. In a recent report, an exacerbation of intestinal tissue destruction has been observed in a model of acute colitis induced by dextran sodium sulphate (DSS) in OPN-null mice (Paes Batista da Silva et al., 2006). These findings are in contrast to the attenuation of inflammatory disease models observed in closed, non-luminal tissues in OPN-null mice, emphasizing the importance of protective functions of OPN in mucosal disease. This review attempts to provide insights into the various known and putative roles of OPN in the complex interplay between the local and immune cell systems involved in mucosal barrier defense, resistance to infection, and tissue repair that are relevant to periodontal, intestinal, respiratory, and urogenital diseases.
**Figure 2.1.** Expression of OPN in inflamed tissues and by inflammatory cells. (A) Immunohistochemical staining for OPN in the normal and inflamed colon. Photomicrographs of sections of the distal colon of 8-week-old mice subjected to experimental colitis were immunostained for OPN and compared with control tissues. Increased staining intensity for OPN is evident in the epithelium and submucosal tissues of the diseased colon (mag. X200). (B) Immunofluorescent staining for OPN (red) and CD44 (green) in isolated neutrophils and macrophages. OPN in neutrophils is present throughout the cells, while CD44 is localized to the periphery of non-polarized neutrophils and in the trailing uropod (green arrowheads) in polarized cells. The distribution of OPN and CD44 is unchanged in CD44−/− and OPN−/− cells. In macrophages, the OPN is seen to co-localize (yellow arrowheads) with CD44 (green arrowheads) in the cell periphery of migrating cells. OPN−/− and particularly CD44−/− macrophages have reduced cell processes and appear more rounded, the OPN in CD44−/− cells being more centrally distributed.
OPN STRUCTURE AND RELATED FUNCTIONS

To understand how OPN has the ability to influence a broad range of biological activities requires an appreciation of its structure and functional motifs (Fig. 2.2). Since several detailed reviews on OPN have been published recently (Giachelli and Steitz, 2000; Sodek et al., 2000; Denhardt et al., 2001a,b; O’Regan, 2003), we include only a brief description of the salient features of OPN and focus on those properties of OPN that are particularly pertinent to mucosal defense. OPN is expressed by a single gene in a cluster of SIBLING family proteins that share structural and functional properties (Fisher and Fedarko, 2003) on the long arm of chromosome 4 in humans and chromosome 5 in mice. OPN is synthesized as a ~ 34-kDa nascent protein that is extensively modified by phosphorylation and glycosylation and sulphation prior to its secretion as a largely unstructured 44- to 75-kDa protein (Sodek et al., 2000). The heterogeneity of the secreted protein reflects differences in these post-translational modifications, which have been related to different functional activities associated with mineralization (Nagata et al., 1989; Sodek et al., 2000) and cell attachment and signaling (Ashkar et al., 2000; Zhu et al., 2001; Suzuki et al., 2002; Weber et al., 2002; Chellaiah et al., 2003). However, most of the known functional activities of OPN can be attributed primarily to highly conserved structural motifs involved in binding mineral and cell-surface CD44 and integrin receptors (Fig. 2.2).
**Figure 2.2.** The locations of 2 integrin-binding sites and 3 CD44 signaling sites are shown (the precise location of the third CD44 signaling motif in the carboxy-terminal thrombin fragment has not been identified). The cryptic SLAYGLR (SLVVGLR in human OPN) integrin-binding site, which is contiguous with the RGD domain, is exposed by thrombin digestion of the adjacent arginine. Thrombin digestion also increases the activity of the RGD. The sites of post-translational modifications are adapted from rat OPN, in which 10–11 phosphorylation sites are modified per molecule. M, methionine; S, serine; L, leucine; D, aspartate; R, arginine; G, glycine; N, asparagine; T, threonine.
Through integrin ligation, OPN can modulate a variety of cellular activities, including cell proliferation, survival, motility, and phagocytosis, all of which involve the cytoskeleton and have an impact on the inflammatory responses of macrophages and the fibrotic activities of fibroblasts. Interactions between the centrally located RGD sequence (arginine-glycine-aspartate) and the αvβ3 integrin, which is highly expressed in macrophages and osteoclasts, have been well-documented. Up-regulation of IL-12 in macrophages is mediated by RGD signaling through the αvβ3 and requires the OPN to be phosphorylated (Ashkar et al., 2000; Weber et al., 2002), as shown also for attachment and signaling in osteoclasts (Chellaiah et al., 2003), and for the migration of osteoclasts (Suzuki et al., 2002) and macrophages (Zhu et al., 2004). In this regard, it is interesting to note that phosphorylation of human OPN occurs in clusters of 3–5 sites, and that these phosphorylation sites are absent from the RGD region, as well as from regions of glycosylation (Christensen et al., 2005). In addition, a second cryptic integrin-binding site is recognized by the α4β1 and α9β1 integrins, which are preferentially expressed by leukocytes and lymphocytes (Bayless et al., 1998; Smith and Giachelli, 1998). This motif is contiguous with the RGD sequence, and, in human OPN, has the sequence "SLVVGLR" ("SLAYGLR" in murine OPN; Fig. 2.2), which is exposed by thrombin digestion of OPN at the conserved arginine, and is considered essential for the development of arthritis in a mouse model (Yamamoto et al., 2003).

CD44 has been identified as a receptor for OPN through which chemotactic (Weber et al., 1996) and cytokine responses of macrophages (Ashkar et al., 2000) have been demonstrated. Activities mediated by the CD44 receptor have implicated the amino-terminal peptide sequence (Fisher and Fedarko, 2003), a cryptic site near a central
thrombin cleavage site (Lin and Yang-Yen, 2001), and an unidentified region in the C-terminal half of the molecule (Weber et al., 2002). The amino-terminal peptide has strong chemotactic activity for macrophages that can be blocked by CD44 antibodies and antibodies to the amino-terminus of OPN (Batista-da-Silva et al., unpublished observations), while Thr 147 in the murine OPN cryptic motif may be important for OPN signaling through the CD44 receptor in B-cells (Lin and Yang-Yen, 2001) (Fig. 2.2). Notably, chemotaxis of macrophages toward formyl-met-leu-phe (fMLP) in vivo is inhibited by antibodies to OPN (Giachelli et al., 1998). However, the interaction between OPN and CD44 may not be direct (Smith and Giachelli, 1998), and, for CD44, variant forms may require non-RGD-dependent mediation by the β1 integrin (Katagiri et al., 1999).

The pleiotropic effects of OPN mediated by the different receptors provide the basis for its emergence as a cytokine that is highly expressed in various pathologies involving inflammation and cancer progression and metastasis, as well as in the pathophysiological processes of wound healing (Giachelli et al., 1998; Liaw et al., 1998; Giachelli and Steitz, 2000; O’Regan and Berman, 2000; Sodek et al., 2000; Lekic et al., 2001; Leali et al., 2003). Moreover, the frequent up-regulation of OPN in a variety of cells in response to a broad range of adverse conditions suggests that it functions as a stress-related glycoprotein. In the immune system, an increased transcription of OPN (identified as early T-lymphocyte activation 1; eta-1) in T-cells (Patarca et al., 1989) was subsequently linked to its suppression of T-lymphocyte function and enhancement of B-lymphocyte proliferation and antibody production (Lampe et al., 1991; Weber et al., 1996). OPN is now recognized as a key cytokine involved in immune cell recruitment and
type-1 (Th1) cytokine expression at sites of inflammation (Ashkar et al., 2000; Chabas et al., 2001; Jansson et al., 2002), and it has been proposed that it be considered a new interleukin family member (IL-28; G Weber, International Conference on Osteopontin and Related Proteins, 3rd ICORP Meeting, San Antonio, May, 2002; Weber, 2002). Macrophages are central to the innate immune response (Fig. 2.3) and are a primary target of OPN. Ligation of CD44 receptors and the β3-integrin by separate domains of OPN attract macrophages and stimulate cytokine and metalloproteinase production, respectively (Weber, 2002). OPN has also been reported to bind and activate pro-stromelysin (pro-MMP3) (Fedarko et al., 2000). Since the expression of this metalloproteinase is increased in wound healing, this surprising activity of OPN may be significant for the resolution of inflammatory diseases.

Recent studies have shown that OPN can bind and recruit complement factor H to cell surfaces through its interaction with the αvβ3 and CD44 receptors (Fedarko et al., 2000). Localization of Factor H to the cell surface is believed to afford protection against immune surveillance and complement-mediated cell lysis, which could have particular significance for the survival of cells at sites of inflammation. The association of OPN with Factor H may also be a way to control OPN pro-inflammatory effects in the various body fluids.
Figure 2.3. Diagram showing possible protective functions of OPN that affect the patency of the epithelium, both directly and indirectly, through the innate immune response to mucosal disease. Noxious agents (bacteria, bacterial products, and antigens) cause epithelial damage, resulting in IL-1 and IL-8 release, which attracts neutrophils and macrophages. This immediate response is controlled by secretion of pro-inflammatory cytokines and the expression of Toll-like receptors (TLRs) and chemokine receptors. OPN expressed by epithelial and immune cells acts as a chemoattractant to macrophages and neutrophils and regulates their phagocytic activity, reactive oxidative burst (ROS) and release of cytokines, and proteolytic enzymes. A protracted innate response will cause further destruction of the epithelial barrier.
In addition to the various secreted forms of OPN, the existence of an intracellular form of OPN (iOPN) that co-localizes with CD44 in migrating fibroblasts, macrophages, and osteoclasts has been reported (Zohar et al., 2000; Suzuki et al., 2002; Zhu et al., 2004). This novel form of OPN—which appears to modulate cytoskeleton-related functions, including cell motility and survival (Zohar et al., 2004), and mediates IFN-α expression in plasmacytoid dendritic cells (Shinohara et al., 2006)—introduces a further layer of complexity in deciphering the roles of different forms of OPN.

**OPN AND INFLAMMATORY DISEASES**

During inflammation, OPN is secreted by T-lymphocytes and activated macrophages and, subsequently, by proliferating fibroblasts and myofibroblasts during granulation tissue formation (Ashkar et al., 2000; O’Regan and Berman, 2000). In the absence of OPN expression, macrophage migration and adhesion are impaired (Giachelli et al., 1998; Zhu et al., 2004), while the ability of OPN to promote fibrosis is consistent with the impaired wound healing in OPN-null mice (Liaw et al., 1998; Jander et al., 2002; Yumoto et al., 2002) and with studies demonstrating that OPN promotes survival of fibroblasts, as well as endothelial cells involved in neovascularization (Denhardt et al., 2001a). The survival-promoting effects of OPN in endothelial cells are mediated by αβ3 activation and signaling through Ras and src tyrosine kinase, followed by activation of NF-κB (Scatena et al., 1998). In fibroblastic cells, the lack of OPN expression leads to caspase-independent necrosis, promoted by oxidants (Zohar et al., 2004). In contrast to programmed cell death, where dying cells undergo phagocytosis and clear the way for
new tissues and cells, necrotic cell death is strongly associated with an exacerbation of inflammation and an increase of tissue destruction. Cell necrosis is a rapid form of cell death, in which damage and rapid permeabilization of cell membranes lead to the release of intracellular contents. The released cellular components act as irritants, recruiting more phagocytes (PMN, macrophages) and aggravating inflammation, and resulting in tissue destruction (Ina et al., 1999; Paleolog, 2003; Kurtovic and Segal, 2004).

In many inflammatory models, the formation of granulation tissue and the intensity of inflammatory reactions are dramatically reduced in the absence of OPN expression (O’Regan et al., 2001). Thus, the development of inflammatory diseases—such as lung (Miyazaki et al., 1995; O’Regan et al., 2001) and kidney (Noiri et al., 1999) fibrosis, arthritis (Yumoto et al., 2002) and multiple sclerosis (Chabas et al., 2001; Jansson et al., 2002)—is markedly suppressed in OPN-null mice. However, the critical role of OPN has been questioned in arthritis and experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, because the development of inflammation and its attenuation in the absence of OPN expression appear to be mouse-strain-specific, and, in some studies, concerns over the removal of flanking genes in back-crossed OPN-null mice have been raised (Blom et al., 2003; Jacobs et al., 2004).

Consequently, the demonstration that arthritis-associated inflammation (Yamamoto et al., 2003) and concanavalin A-induced hepatitis (Diao et al., 2004) can be suppressed by antibodies directed at the cryptic "SLAYGLR" sequence in mice provides important alternative evidence of the pivotal role of OPN in the inflammatory response, by an approach that is not subject to compensatory mechanisms that can arise in knock-out mice. Importantly, in all of these pathologies, OPN expression by activated macrophages,
T-cells, and fibroblastic cells is increased as part of the inflammatory response and in the subsequent repair process (Fig. 2.1), which also supports the significant contribution OPN makes in the progression and sequelae of inflammatory diseases. High levels of OPN expression are also a hallmark of monocyctic granulomatous reactions in the context of tuberculosis and silicosis (Nau et al., 1999; O’Regan et al., 1999).

While most studies have generally concluded that OPN is a key regulator of cell-mediated inflammation, the potential role of OPN in protecting tissues from disease and/or excessive inflammatory destruction has largely been ignored. The apparent detrimental effects of OPN relate to its pro-inflammatory effects on macrophages, which respond by increased production of the Th1 cytokines IL-12 and IFN-γ and decreased production of the Th2 cytokine IL-10, thereby polarizing the immune response to the Th1 pathway (Ashkar et al., 2000). These combined effects promote a protracted inflammation that is characteristic of autoimmune diseases, in which OPN may also contribute prominently (Lampe et al., 1991; Ashkar et al., 2000; Chabas et al., 2001). Notably, in OPN-null mice, the Th1 cytokines are reduced, while the Th2 cytokines are elevated. The protective effects of OPN may be more evident in mucosal inflammation, in which the patency of an epithelial lining is important for mediating injury and antigen presentation. However, while skin wound repair has been analyzed in OPN-null mice (Liaw et al., 1998), few studies have examined the role of OPN in mucosal disease. Following the creation of incision wounds in skin, the expression of OPN is increased within 6 hrs. However, in the absence of OPN expression, matrix production is impaired, and cell debris accumulates at the wound site (Liaw et al., 1998). Since neutrophil infiltration is an immediate response to mucosal injury that leads to the development of
an acute inflammation, these observations suggest that, in OPN-null mice, there is an increased destructive activity of neutrophils due to slow clearance by macrophages, which may also have reduced phagocytic activity. In addition to the delayed resolution of the innate immune response, fibroblast function also appears to be compromised. This interpretation is consistent with the increased tissue destruction and markedly elevated (Fig. 2.1) PMN activity that we have observed in an acute colitis model in OPN-null mice, in which little epithelial regeneration is observed during remission in the absence of OPN expression (Paes Batista da Silva et al., 2006).

**DETERMINANTS OF MUCOSAL IMMUNITY**

A specialized epithelial barrier lining the oral cavity, the intestinal tract, respiratory tract, and urogenital systems provides the primary protection for mucosal tissues. Breakdown of the barrier functions leads to the infiltration of bacteria or luminal noxious agents that cause inflammatory diseases, including periodontal disease in the oral cavity, inflammatory bowel diseases (IBDs) (Tlaskalova-Hogenova et al., 2004), respiratory diseases (Delclaux and Azoulay, 2003), and urogenital diseases (Connell et al., 1997; Mulvey, 2002). Physical protection is achieved by the formation of tight junctions connecting the epithelial cells, while goblet and other specialized secretory cells produce mucous enriched with antimicrobial glycoproteins, IgA class antibodies, cytokines, and chemokines to defend against the invasion of pathogens and maintain the integrity of the epithelial barrier (Mowat, 2003; Acheson and Luccioli, 2004). Another
mechanical advantage of this barrier is the viscosity of the mucous secreted by the epithelial cells; this can prevent the adherence of particles or micro-organisms that are otherwise expelled by ciliary movement in the respiratory tract, or by peristalsis in the gut. Failure of these barrier functions may lead to recurrent respiratory tract infections or infestation and infection of the gut lumen, respectively.

Noxious irritation/damage of the epithelial barrier can be mediated by epithelial cells through special recognition receptors such as Toll-like receptors, by specialized epithelial cells (microfold; M cells), or, more directly, by the penetration of bacteria or their products (Otte et al., 2003; Shi and Walker, 2004). Penetration of the epithelial barriers results in activation of resident neutrophils (polymorphonuclear leukocytes; PMNs) and macrophages; these are professional phagocytes which provide the "immediate innate immune response" and non-specifically engulf foreign material and bacteria (Fig. 2.3). Cytokines and chemokines released from the injured epithelium as well as the leukocytes increase neutrophil and macrophage infiltration into the tissue, and thereby initiate the inflammatory response. Long-term residents of the subepithelial tissue, the antigen-presenting cells (APC)/dendritic cells, which are specialized macrophages, initiate the more specific "adaptive immune responses". The adaptive system is based on a specific recognition between APC and naïve T-cells, resulting in the differentiation of Th1, T-helper, and cytotoxic (NKT) T-cells, which control the immune reaction. Notably, many studies have correlated OPN expression with epithelial barrier changes (Gassler et al., 2002), with macrophage (Giachelli and Steitz, 2000; O’Regan et al., 2001; Sodek et al., 2002; Zhu et al., 2004), neutrophil (Alstergren et al., 2004), and
lymphocyte activities (Ashkar et al., 2000), and with the function of reparative fibroblasts (Sodek et al., 2002).

**OPN AND THE EPITHELIAL BARRIER**

OPN has been recognized as an important luminal regulator (Brown et al., 1992), due to its expression by epithelial cells covering luminal cavities capable of active secretion and absorption of nutrients or gasses. Indeed, earlier studies showing that epithelial cells secrete OPN first indicated that OPN is involved in controlling epithelial barrier permeability and secretory functions (Butler, 1989).

In the gastrointestinal tract, a layer of columnar epithelial cells separates the underlying mucosa from the lumen and provides a reservoir for macrophages and T- and B-lymphocytes, which are concentrated in an organized subepithelial network along the gut and can be identified in focal areas, such as the Peyer’s patches in the distal small intestine. Moreover, the epithelial layer also contains specialized cells, such as the microfold (M) cells, capable of recognizing specific bacteria and antigens and transferring them to specialized APCs that reside in the vicinity of the epithelial layer (Neurath et al., 2002a; Mowat, 2003; Acheson and Luccioli, 2004). The MHC class-II and Toll-like receptors on specialized epithelial cells may be involved directly with antigen presentation to underlying CD4+ T-cells (Acheson and Luccioli, 2004) (Figs. 2.3, 2.4). In recent studies, it has been suggested that the constitutive expression of OPN by epithelial cells (Fig. 2.1) is required for maintaining the epithelial barrier in the intestines (Gassler
et al., 2002), and as a defense against tubulo-interstitial destruction in kidney diseases (Sibalic et al., 1997; Rangan et al., 2001). Moreover, increased OPN expression by tubular epithelium has been shown to have a critical role in mediating monocyte infiltration into the interstitium, leading to tubulo-interstitial alterations secondary to primary glomerulonephritis (Okada et al., 2000). OPN also has the potential to regulate specific functions of epithelial cells involved in the barrier defense process (Fig. 3), such as the expression of MHC-II and Toll receptors, which aid in antigen presentation and inflammatory signaling (Mowat, 2003; Basu and Fenton, 2004; Iwasaki and Medzhitov, 2004). For example, while the physical epithelial barrier can arrest microbial infection of mucosal surfaces by *Candida* (Steele et al., 2000), the Toll-like receptor 2 is required for the innate host defense against the spirochete *Borrelia burgdorferi* (Wooten et al., 2002).

In airway systems, defensins secreted by neutrophils, originally thought to be important only for killing bacteria, can induce the differentiation of epithelial cells into mucosal-lining cells, which are characterized by their mucin secretion (Aarbiou et al., 2004) and enhancement of wound repair. Epithelial enterocytes in the gastrointestinal tract lack the typical microvilli associated with mucous-secreting cells, associate specifically with invading bacteria for transfer to adjacent dendritic cells, and take part in the initiation of the adaptive defense process (Debard et al., 2001; Mowat, 2003) (Fig. 2.4).

The ability of the epithelial barrier to resist stress and trauma, and to regenerate, is important for the subsequent repair of the diseased tissues. Death of epithelial cells, therefore, is an important event associated with mucosal damage and occurs through up-regulation of the Fas ligand, and in response to TGF-β1 expression and an increase in oxidative burst (Barkla and Gibson, 1999; Ophascharoensuk et al., 1999; Hagimoto et al.,...
In view of the role of OPN in cell survival, its presence may be important for supporting programmed cell death and preventing rapid necrotic death, which can result in intense inflammation and loss of epithelial barrier and protection.

**OPN IN MUCOSAL INFLAMMATION**

The increased expression of OPN at sites of inflammation by epithelial, stromal, and immune cells is consistent with functions involving both the innate and adaptive pathways (Figs. 2.3, 2.4).

Innate immunity (Fig. 2.3) is the first line of defense for the broken epithelial barrier. IL-8 released from the injured epithelial cell recruits neutrophils and macrophages, which are capable of non-specific and specific phagocytosis—the macrophages utilizing mannose receptor or the CD14 (Toll 4) receptor for lipopolysaccharide. Upon phagocytosis, neutrophils and macrophages will continuously produce oxidative products such as hydrogen peroxide and nitric oxides. These cells, especially the macrophages, release mediators, which amplify the inflammatory reaction. The mediators include TNF-α, IL-1, MCP-1, Rantes, and TGF-β1, and degradative hydrolytic enzymes responsible for connective tissue degradation, such as the matrix metalloproteinases (MMPs). Other immediate changes associated with the innate immune response occur in response to increased blood flow to the area, with a local decrease in blood velocity. This is accompanied by the expression of adhesion molecules (e.g., VCAM-1) on the endothelial cells, which increases adhesion of leukocytes and vessel permeability, thereby facilitating extravasation of leukocytes to the inflamed tissues.
Osteopontin in Adaptive Immunity

Mucosa-Associated Lymphoid Tissues (MALT)

OPN

Regulatory T(Th3)

TGF-β1

Matrix Remodeling

Th2

Th1

IL-4

IL-10

NKT

CTL/CD8

IFN-γ

Cell-mediated Response

B Cell

IgA Secretion

B Memory

OPN

MHC II

TCR

APC

MHC II

T Memory

IL-12

MIF

PMN

Oliver

MyoFb
**Figure 2.4.** A diagram indicating how osteopontin may influence the adaptive immune response in mucosal tissues. Antigen taken by APC/dendritic cells and presented to naïve T-cells/CD4+ results in T-cell proliferation and differentiation into Th1 cells. The Th1 cells release IL-12, IFN-γ, MIF, OPN, and TNF-α, which recruit macrophages for further activation of Th1 cells and promote the further recruitment of neutrophils and natural killer cells (NKT). Th2 release of IL-10 and IL-4 increases B-cell differentiation, attenuates macrophage activation, and, in conjunction with regulatory T-cells, initiates repair by myofibroblasts. Following removal of noxious agents, reduced inflammation will allow deposition of new matrix by myofibroblasts, leading to epithelial regeneration. OPN can modulate the inflammatory reaction and promote repair through its effects on T-cell differentiation and by its ability to influence the survival of epithelial cells, macrophages, and fibroblasts.
mediated by their integrin receptors, LFA-1 and Mac-1. The effect of innate immunity on the inflammatory progression can be demonstrated in glomerulonephritis models, in which OPN expression is increased (Eddy and Giachelli, 1995; Heinzelmann et al., 1999; Kitching et al., 2002), together with the up-regulation of ICAM-1s IL-1, TGF-β1 MCP-1, and IL-8, and a decrease in IL-10 expression.

The short-lived immune response protects the injured organ prior to the development of the adaptive response (Fig. 2.4). The presentation of antigen by differentiated macrophages to naïve T-cells stimulates the differentiation of lymphocytes, including memory, helper, regulatory, and cytotoxic (NKT) T-cells, which augments further macrophage activity, including the clearance (phagocytosis) of apoptotic PMNs (Haslett et al., 1994). In response to IL-4, Th2 cells, in co-operation with Th1 cells, stimulate proliferation and differentiation of antibody-producing B-cells, while IL-10 secreted by Th2 cells regulates Th1 activity and thereby indirectly controls macrophage activity. During controlled infection, regulatory T-helper cells secrete IL-4 and TGF-β1, which regulate the activity of the effector lymphocytes and stimulate fibroblast proliferation required for tissue remodeling (Fig. 2.5). Notably, increased secretion of TGF-β1 by fibroblasts stimulates the proliferation of effector T-cells as well as antibody-secreting B-cells. In this respect, the activation of the adaptive response is more significant in mucosal defense, since mucosal tissues are challenged continuously by pathogens and noxious agents. The adaptive immune system is important in mucosal immunity, as well as in maintaining tolerance toward resident flora and nutrition products. Stimulation of lymphocyte growth and differentiation is achieved through the stimulation of the dendritic cells, which migrate to lymph-associated areas, such as the mucosal-
associated lymphoid tissues (MALT; Fig. 2.4), and not in the infected inflamed area.

Differentiated effector T-cells express selectins, which help them target the inflamed area and extravasate in a similar fashion to the PMNs. Migrating T-cells will be predominantly Th1 cells, which will stimulate further macrophage and T-cytotoxic cell activities.

OPN expression has been primarily shown to regulate the cell-mediated part of immunity (Ashkar et al., 2000). OPN secreted by activated T-cells attracts macrophages and stimulates Th1 cytokine release through ligation of the αvβ3 receptor, while the secretion of the Th2 cytokine IL-10 is suppressed by OPN signaling through the CD44 receptor. In the absence of OPN expression, the Th2 cytokine response is predominant, and OPN-null mice display an increased susceptibility to intracellular pathogens, including *Rickettsia tsutsugamushi*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and herpes simplex virus-type 1 (Patarca et al., 1993; Nau et al., 1999; Ashkar et al., 2000). The impact of bacterial infection is particularly relevant in mucosal immunity. For intracellular pathogens, the generation of a cytotoxic T-cell response is critical, since the immune system faces a challenge in that the pathogens are hidden within host cells. Resistance to *Rickettsia* infection is mediated by a marked induction of OPN, leading to an early monocyte influx into infected sites and rapid acquisition of macrophage bactericidal activity. Susceptibility to *Rickettsial* infection, in contrast, reflects delayed and weak OPN responses and is characterized by an early accumulation of neutrophils at sites of infection. However, because extracellular pathogens, such as the spirochete *B. burgdorferi*, express lipoproteins that are highly immuno-stimulatory and pro-inflammatory, the requirement of an OPN-mediated stimulation of the Th1 response is circumvented (Craig-Mylius et al., 2005). Despite the direct effects of OPN on T effector
cells, its importance, especially in mucosal immunity, may be mediated through the control of differentiated macrophage (i.e. dendritic cells) activity (Figs. 2.3, 2.4). OPN has been shown to affect macrophage activities such as chemotaxis, phagocytosis, bone resorption, cytotoxic activity, granuloma formation, and fibrotic repair (Denhardt et al., 1995; Miyazaki et al., 1995; McKee and Nanci, 1996; Rollo and Denhardt, 1996; Rollo et al., 1996; Giachelli et al., 1998; Zohar et al., 2000; O’Regan et al., 2001; Ide et al., 2003; Zhu et al., 2004; Ogawa et al., 2005). Different macrophage progeny are needed for the innate immunity response, functioning as unspecific phagocytic cells, but perhaps more importantly as dendritic (APC) cells (Figs. 2.3, 2.4). The dendritic cells signal the activation of lymphocyte proliferation and their differentiation into specific immune-effector cells (i.e., T-cell subclasses and B-cells), pro-inflammatory soluble mediators, and, eventually, myofibroblasts, matrix, and repair. Thus, a lack of macrophages or a malfunction in monocyte differentiation will result in an impaired specific immune response and/or repair.

Notably, OPN may not be needed for basic activity of isolated lymphocytes or macrophages in culture, but may be required for their interaction and co-operative effects in promoting the transition from innate to adaptive responses and the initiation of repair (Fig. 2.5).
Figure 2.5. A simple depiction of the role of OPN in the repair of submucosal connective tissues. Secreted OPN (sOPN), produced mainly by macrophages and Th1 cells, signals through CD44 and integrin receptors on fibroblasts, which differentiate into myofibroblasts. An intracellular form of iOPN associated with CD44 modulates cytoskeleton-related activities, including migration in macrophages (MØs) and fibroblasts, and both sOPN and iOPN produced by myofibroblasts (MyoFb). During the reparative phases, OPN signals through αvβ3 and/or CD44 receptors to stimulate proliferation and differentiation of fibroblasts and protect them from apoptosis, thereby promoting matrix deposition and mucosal repair.
Neutrophils (PMNs)

Also termed granulocytes or PMNs, the neutrophils provide the immediate and non-specific line of defense for broken mucosal surfaces. The release of cytokines such as IL-8 by damaged epithelial cells recruits PMNs to sites of injury to initiate the innate defense. In some situations, mucosal immunity may involve defense through the epithelial and PMN cells only, such as in the arrest of superficial Candida infections (Steele et al., 2000; Schaller et al., 2004). PMNs which attack invading bacteria or foreign material release cytotoxic products that, unless controlled, may result in destruction of the host tissue (Jaeschke and Smith, 1997; Heinzelmann et al., 1999). The infiltration of the subepithelial tissues by PMNs is an important stage in the inflammatory process of mucosal damage in inflammatory bowel diseases, such as Crohn’s disease and ulcerative colitis (Nikolaus et al., 1998; Ina et al., 2002; Kruidenier et al., 2003; Le’Negrate et al., 2003; Kurtovic and Segal, 2004). In normal self-restricting mucosal defense processes, PMNs are usually cleared within 24 hours of injury, due to the invading macrophages, which engulf apoptotic neutrophils and arrest further neutrophil mobilization and tissue destruction (Haslett et al., 1994; Hart et al., 2000). The lack of neutrophil clearance by macrophages may result in the persistence of PMNs in inflamed tissues, which can result in increased destruction (Batista-da-Silva et al., submitted). That a similar situation is seen in TNFα-null mice, in which experimental colitis is aggravated due to hyperactivation of neutrophils (Naito et al., 2003), is of particular interest, in that TNFα expression is suppressed in response to DSS-induced colitis in OPN-null mice (Paes Batista da Silva et al., 2006).
While the release of OPN by neutrophils can contribute to the recruitment of macrophages, few studies have reported on OPN expression by neutrophils. Chitosan and G-CSF have been shown to increase OPN mRNA expression in PMNs, although the increase in the release of OPN into the medium was modest (Ueno et al., 2001). Immunocytochemical staining of OPN in PMNs (Fig. 2.3) confirms the presence of OPN throughout the cytoplasm and, in contrast to macrophages, shows no particular association with CD44, which is characteristically concentrated in the uropods of polarized PMNs. Also, in contrast to macrophages (Zhu et al., 2004), the cell-surface expression of CD44 is not influenced by OPN, which may reflect differences in the migratory characteristics of these cells (Alstergren et al., 2004).

**Macrophages**

Macrophages participate in both immediate innate and adaptive immune responses (Figs. 2.3, 2.4). Differential expression of OPN by macrophages and T-cells determines the relative levels of the immediate and delayed-type hypersensitivity responses, which are neutrophil-dependent and neutrophil-independent, respectively. In immediate responses, the macrophages are attracted and activated by cytokines secreted by neutrophils, thereby promoting a protracted inflammatory reaction that frequently results in excessive fibrosis and scar formation (Fig. 2.5). Macrophages, therefore, may be the most versatile cells in mucosal protection. Macrophages infiltrating the injured mucosal tissues in early stages help the PMNs phagocytose invading pathogens, while controlling the amount of damage by PMNs and attracting the more specific adaptive pathways of
immune cell-mediated protection of lymphocytes. Notably, in healthy subepithelial
tissues of the gut, respiratory tract, and the urogenital systems, differentiated
macrophages reside for long periods as dendritic cells, which respond to noxious insults
by initiating the adaptive immune response. As noted previously, not only do the
macrophages/APCs in mucosal surfaces activate the adaptive immune response, but they
also maintain immune tolerance toward non-pathogenic normal flora (Mowat, 2003). The
cell-mediated immune response is characterized by the formation of granuloma tissue,
which occurs during wound healing, and, consistent with the effects of OPN on
macrophage function, granuloma formation is impaired in OPN-null mice (O’Regan et
al., 1999, 2001; Ashkar et al., 2000; Morimoto et al., 2004).

Macrophages produce large amounts of OPN, which is further up-regulated by
LPS stimulation (Gao et al., 2004). The OPN produced by macrophages may act as an
opsonin, facilitating phagocytosis (McKee and Nanci, 1996), as well as cell adhesion,
through RGD binding. While immunostaining of OPN displays a perinuclear distribution
typical of secreted proteins, in migrating macrophages, OPN is also seen co-localizing
with CD44 at the cell membrane of cell processes (Fig. 2.5) as an intracellular form of
OPN (iOPN). The iOPN and its association with CD44 were originally identified in
migrating fibroblasts (Zohar et al., 2000) and subsequently characterized by pulse-chase
labeling studies in macrophages (Zhu et al., 2004). In the absence of OPN expression, the
formation of cell processes associated with the migration of macrophages and their
chemotaxis to fMLP and MCP-1, which act through G-protein-coupled receptors, are
impaired. The impairment appears to be related to decreased expression of CD44 (Zhu et
al., 2004), which is regulated by OPN functioning through the αvβ3 integrin in
macrophages (Marroquin et al., 2004) and osteoclasts (Chellaiah et al., 2003) and has been shown to be crucial for the polarization and migration of neutrophils (Alstergren et al., 2004).

In addition to its effects on macrophage recruitment and activation, MCP-1 also has important effects on OPN expression and cytokine synthesis by macrophages that affect myofibroblast accumulation in healing infarcts (Dewald et al., 2005). In macrophages stimulated with LPS and IFN-γ, nitric oxide (NO) directly up-regulates endogenous OPN, which acts as a negative feedback regulator of iNOS to reduce nitric oxide synthesis (Takahashi et al., 2000; Guo et al., 2001; Speyer et al., 2003). Recent studies have shown that the development of atherosclerosis is prevented by Liver X Receptor (LXR) ligands, which inhibit cytokine- and LPS-induced OPN expression in macrophages by targeting an AP1-element in the OPN promoter (Ogawa et al., 2005). Thus, expression of OPN in macrophages is required not only for recruitment, but also to orchestrate the specific adaptive and non-specific innate defense pathways.

**Lymphocytes**

OPN is considered to be an important lymphocyte mediator secreted by activated T-lymphocytes that induces macrophage migration and suppresses the production of reactive oxygen species, while enhancing immunoglobulin production and proliferation of B-lymphocytes (Weber et al., 1996). In the original studies of lymphocytes, OPN was identified as a T-lymphocyte activation-1 (Eta1) cytokine (Patarca et al., 1993) that was
subsequently shown to be required for cell-mediated immunity and the development of the Th1 pathway and macrophage activity (Ashkar et al., 2000; Chabas et al., 2001; Jansson et al., 2002; O'Regan, 2003). The function of tissue resident macrophages/dendritic cells is not only to destroy pathogens but also to mediate the adaptive immune response by carrying pathogen antigens through the lymph circulation to peripheral lymphoid organs, where they present them to naïve lymphocyte precursors through specialized MHC receptors (Fig. 2.4). Failure of dendritic cell activation may result in improper adaptive immune response or immune-tolerance to antigens.

Lymphocyte function in mucosal protection (Figs. 2.3, 2.4) is part of the more specific adaptive immunity. Lymphocyte activation and differentiation will usually occur via two routes:

1. Differentiation of specialized T-cells starts primarily with co-stimulation of the antigen presented by the dendritic cells through MHC-II and Toll-like receptors to naïve T-cells, but also in response to chemokines and cytokines released by dendritic cells, such as IL-12 and CC-chemokine ligand.

2. In the lymphoid tissue, there is a specialized macrophage population, which interacts with antigen-specific receptors of B-cells with co-stimulation of differentiated T-cells. B-cells then differentiate into their effector cells (i.e., plasma cells), proliferate, and produce antibody, mainly IgA-class immunoglobulins, which can be secreted through the epithelial barrier for mucosal defense. Most of the subepithelial lymphocytes are antibody-secreting B-cells, plasma cells, and memory T-cells. Effector T-cells, which are able to destroy
infected cells and activate other cells of the immune system, are CD4+ cells, which include most of the T-helper subtypes, while some are CD8+ cells representing T killer (NKT) cells, which have a cytotoxic function and secrete IFN-$\gamma$ to encourage killing by macrophages (Mowat et al., 2003; Watford et al., 2003; Dakic et al., 2004; Nagler-Anderson et al., 2004).

In CD4+ T-cells, OPN mRNA is expressed in Th1, but not in Th2, polarized cells (Nagai et al., 2001). OPN promotes adhesion of activated T-cells, and this activity is enhanced following proteolytic cleavage of OPN by thrombin (O’Regan et al., 1999). At low concentrations, OPN promotes chemotaxis but not chemokinesis of T-cells, while activated T-cell adhesion is enhanced at higher concentrations, especially following thrombin cleavage of OPN (O’Regan et al., 1999). OPN also co-stimulates T-cell proliferation and increases CD3-mediated T-cell production of IFN-$\gamma$ and CD40 ligand (O’Regan and Berman, 2000). At low concentrations, OPN promotes chemotaxis, but not chemokinesis, of T-cells. However, this response is inhibited at higher OPN concentrations.

A variety of inflammatory and autoimmune diseases—including multiple sclerosis, rheumatoid arthritis, and atherosclerosis—are critically regulated by NKT cells, which also secrete OPN. The OPN augments NKT cell activation and triggers neutrophil infiltration and activation. Consistent with this role of OPN in NKT cell function, OPN-null mice, similar to NKT cell-deficient mice, are refractory to Con A-induced hepatitis. However, a neutralizing antibody, specific for a cryptic integrin-binding epitope of OPN that is exposed by thrombin cleavage, ameliorates the development of hepatitis in normal
mice (Diao et al., 2004). In contrast, over-expression of OPN in the liver of transgenic mice resulted in massive liver necrosis and monocyte infiltration, due to an imbalance of the Th1 and Th2 immune responses (Mimura et al., 2004), presumably caused by the attraction of macrophage precursors by OPN and its promotion of the Th1 response.

Impaired OPN−/− macrophage differentiation and functions (Weber et al., 2002; Zhu et al., 2004) may be responsible for deficient healing in OPN-null mice (Liaw et al., 1998; Rittling et al., 1998). OPN induces T-cell proliferation, adhesion, and chemotaxis, especially in relation to granulomatous lesions (O’Regan et al., 1999). Moreover, soluble OPN may modulate the differentiation and proliferation of CD4+ and CD8+ lymphocytes (Higuchi et al., 2004). Over-expression of OPN in transgenic mice increased the numbers of CD4+ T-cells, but not CD8+ T-cells, in the spleen, particularly in the lymph nodes. Skin sensitization with 2,4-dinitrofluorobenzene (DNFB) in these mice increased the number of CD4+ cells and recruitment of CD8+ cells. However, peritoneal sensitization with DNFB (Higuchi et al., 2004) resulted in increases in the numbers of CD8+ T-cells in the peritoneal exudate, with no difference in the numbers of CD4+ T-cells. These results suggest that different responses might be anticipated in mucosal immunity, where there is an epithelial barrier involvement in the transfer of the inflammatory signals. In this regard, studies of colitis in OPN-null mice support the suppression of CD4+ and CD8+ cell numbers of the inflamed OPN-null spleens (Paes Batista da Silva et al., 2006). These observations also suggest a direct effect of OPN on T-cells, or, alternatively, indirect effects due to lack of functional macrophages or differentiated dendritic cell stimulation. This is accompanied by a decrease of IL-12, IFN-γ, and the oxidative burst necessary for the development of proper protective inflammatory reaction.
IFN-γ treatment of Th-1 cells has been shown to induce OPN mRNA and protein expression in a time-dependent and dose-dependent manner (Li et al., 2003), consistent with the contribution of OPN to Th1 activation. These effects may also be dependent on the ability of macrophages to differentiate into dendritic cells necessary for the T-cellular response (Weiss et al., 2001) and their mobilization from the regional lymph nodes. Upregulation of CD40L by OPN in T-lymphocytes (O’Regan and Berman, 2000) provides mechanistic support for the association of OPN with polyclonal B-cell proliferation and humoral autoimmune disease (Weber et al., 1996). During stimulation of the lymphoid centers, specific dendritic cells, through interaction with CD4 (naïve) lymphocytes, present antigen to B-cells, which have antigen-specific receptors that enable them to internalize large amounts of specific antigens, proliferate, and produce specific antibodies. Interestingly, an important mechanism for IgA production is that promoted by Th3 secreted TGF-β1, which controls IgA switching and secretion in vivo (Borsutzky et al., 2004). B-cells then migrate into the surrounding mucosa and release secretory IgA through the epithelial lining of the mucosa. The secretory IgA binds directly to pathogens and prevents their attachment to epithelial cells; it also coats antigens, promoting their engulfment by macrophages. Although it is unclear how TGF-β1 and its associated receptors control B-cell activity, the Toll death receptors and G-protein-coupled receptors appear to be involved (Cazac and Roes, 2000; Roes et al., 2003).

TGF-β1 and OPN are important mediators of extracellular matrix formation and wound healing (Bendeck et al., 2000; Sodek et al., 2000; Denhardt et al., 2001a,b), and the matrix-promoting and immunoregulatory activities of TGF-β1 have been studied quite extensively (Kitani et al., 2003; Wahl and Chen, 2003). However, the role of OPN and its
relation to TGF-β1-mediated activities are largely unknown. TGF-β1 modulates
differentiation of naïve CD4 lymphocytes (Wang and Mosmann, 2001), and over-
expression of TGF-β1 reduces the susceptibility of mice to colitis by blunting Th3
immune and humoral responses (Egger et al., 1998). TGF-β1 expression during
inflammation and immune reactions also stimulates IL-10 secretion in Th1 cells,
regulates T-lymphocyte activity, and induces OPN expression in immune as well as
reparative fibroblasts (Overall et al., 1991) (Fig. 2.5).

**OPN AND THE INITIATION OF HEALING**

Monocytes/macrophages, lymphocytes, and mast cells recruited to the submucosa
produce the cytokines and growth factors necessary for fibroblast proliferation and neo-
vascularization, as part of granulation tissue formation (Orlando, 2002). Activated
macrophages are responsible for the oxidative burst and for IL-10 secretion—processes
that up-regulate fibroblast proliferation and differentiation into myofibroblasts (McKaig
et al., 2002) through the production of TGF-β1 (di Mola et al., 1999; McKaig et al.,
2002). Connective tissue remodeling occurs around the ulcerated epithelium with the
formation of granulation tissue. Matrix metalloproteinases (MMPs) derived from
macrophages and from the activated fibroblasts (Vaalamo et al., 1998; Pirila et al., 2003)
augment matrix remodeling and overall tissue destruction (Fig. 2.5). OPN has also been
reported to activate MMP-3 (Fedarko et al., 2000), the expression of which is increased
markedly in inflamed colons of patients with ulcerative colitis and Crohn’s disease, and
correlates with the loss of mucosal integrity, suggesting an important role for stromelysin
and its possible activation by OPN in the process of destruction and tissue remodeling in
inflammatory bowel diseases (Heuschkel et al., 2000; von Lampe et al., 2000). Myofibroblasts, which are typically associated with reparative granulation tissue formation (Desmoulière, 1995; Neubauer et al., 2001), are characterized by an enhanced expression of α-smooth muscle actin (α-SMA) in stress fibers (Jelaska and Korn, 2000; Thibault et al., 2001; Tomasek et al., 2002; McKaig et al., 2003). During matrix formation, myofibroblasts respond to TGF-β1 by increased expression of extracellular matrix (ECM), including collagen, and selective suppression of MMPs (Overall et al., 1991). Since TGF-β1 also increases α-SMA, it is conceivable that the myofibroblast phenotype induced in fibroblasts is mediated through the up-regulation of TGF-β1, which also increases the expression of OPN, integrins, and α-actinin (Kawano et al., 2000; Mazzali et al., 2002) and promotes integrin-mediated collagen gel contraction (McKaig et al., 2003). TGF-β1 and OPN also down-regulate apoptosis of fibroblasts and thus increase matrix deposition (Desmoulière, 1995; Jelaska and Korn, 2000; Zohar et al., 2004). Indeed, connective tissue wound healing and fibrosis, subsequent to inflammatory disease, are impaired in OPN-null mice (Liaw et al., 1998; Trueblood et al., 2001).

That OPN is expressed by epithelial and mucosal cells, as well as by immune cells in the intestines, has been shown (Qu and Dvorak, 1997; Gassler et al., 2002). However, despite the perceived importance of OPN in cell-mediated immune responses and wound healing, there have been few studies of OPN in the normal or diseased intestine. In support of the proposed functions of OPN in protecting the intestine from pathogens and in the development of inflammatory bowel diseases (Masuda et al., 2003), our preliminary studies indicate that destruction of the intestinal tissues is exacerbated in OPN-null mice subjected to experimental colitis (Paes Batista da Silva et al., 2006). The
loss of mucosal integrity and inflammatory destruction as seen in patients with ulcerative colitis and Crohn’s disease correlates with the production of IL-1β, IL-6, TNF-α, IL-10, TGF-β1, and activated MMPs (Fedarko et al., 2000; Heuschkel et al., 2000; von Lampe et al., 2000; McKaig et al., 2003). Impairment of these mediators’ activity and overall granulation tissue formation will alter the process of tissue remodeling and repair in inflammatory bowel diseases.

**SUMMARY**

The multi-functional role of OPN in inflammatory diseases is now well-established. However, elucidating these functions is complicated by the temporo-spatial expression of different forms of OPN by inflammatory cells and reparative fibroblasts, as the different immune system pathways respond in conjunction with reparative cells to protect and repair damaged tissues. In mucosal disease, further complexity is introduced by the expression of OPN by most of the sub-mucosal cells, including epithelial, immune cells, and fibroblasts, all of which contribute to the barrier function, immunity, and repair processes. Given the pivotal role of OPN in mucosal protection, identifying the specific functions of the different signaling motifs in the context of the different isoforms of OPN remains a challenge in ongoing studies of mucosal diseases.
CHAPTER 3: Exacerbated tissue destruction in DSS-induced acute colitis of OPN-null mice is associated with downregulation of TNF-α expression and non-programmed cell death


ABSTRACT

Osteopontin (OPN), a pro-inflammatory mediator, is constitutively expressed in normal gut and is upregulated in inflammatory colitis. To determine the significance of OPN in inflammatory bowel disease, we studied the development of acute, experimental colitis induced by dextran sulfate sodium (DSS) in OPN-null and wild-type (WT) mice. OPN expression was markedly increased in WT diseased colons, while a higher disease activity index, including spleen enlargement, bowel shortening, and mucosal destruction, was observed in OPN-null mice. Although peripheral blood neutrophil numbers were lower in DSS-treated OPN-null mice, tissue myeloperoxidase levels, reflecting enhanced neutrophil activity, were increased in the diseased colons. In comparison, lymphocyte numbers in peripheral blood were increased earlier than in DSS-treated WT mice. Despite a significantly greater spleen enlargement, flow cytometric analysis of splenocytes from the DSS-treated OPN-null mice revealed lower numbers of differentiated macrophages and (CD4+ and CD8α+) lymphocytes. Whereas pro-inflammatory cytokines, including G-CSF, RANTES, MIP1α, and TNF-α, were increased <10-fold in DSS-treated WT splenocytes, expression of these cytokines was dramatically suppressed in the DSS-treated OPN-null splenocytes as well as gut tissues. The suppressed TNF-α response in OPN-null mice was reflected in a marked increase in non-apoptotic cell death in diseased colons. Collectively, these studies demonstrate that OPN is required for mucosal protection in acute inflammatory colitis.
INTRODUCTION

Ulcerative colitis and Crohn's disease are inflammatory bowel diseases (IBDs) with a high prevalence in children and adult (Bland et al., 1999; Kugathasan et al., 2003; Kim and Ferry, 2004) that are characterized by profound destructive effects that occur during cyclical periods of exacerbation and remission. The destructive lesions involve chronic inflammation, mucosal damage, and epithelial cell destruction (Ardizzone and Porro, 2002; van Deventer, 2002; Staros, 2003). Although the etiology of IBD is unknown, it is thought to involve susceptible hosts with dysregulated immune responses to microbial and dietary antigens (Orlando, 2002). TNF-α, oxygen radicals and matrix metalloproteinases produced by neutrophils and activated macrophages contribute to the epithelial ulceration and sub-mucosal destruction. Th1 cells produce pro-inflammatory mediators (i.e., IFN-γ, IL-12, IL-18, IL-2, TNF-α, NF-κB, and OPN) (Wittig et al., 2000; Gassler et al., 2002) and dominate in the inflamed tissues longer than their controlling Th2 cells (Shetty and Forbes, 2002; Wittig et al., 2002).

Osteopontin (OPN), a matricellular glycoprotein that exhibits a broad range of functional activities in physiological and pathological processes (Sodek et al., 2000; Denhardt et al., 2001b), is upregulated in epithelial, mesenchymal, and inflammatory cells during inflammation and wound healing (O'Regan and Berman, 2000). OPN has been recognized as a key cytokine involved in immune cell recruitment and cell mediated immunity (Ashkar et al., 2000; Chabas et al., 2001; Jansson et al., 2002). OPN is secreted by T-lymphocytes and activated macrophages, and subsequently by proliferating fibroblasts, during reparative matrix formation (Ashkar et al., 2000; O'Regan and Berman, 2000). In mice with a targeted disruption of the OPN gene, the development of
the inflammatory response in animal models of arthritis, kidney, and lung diseases is attenuated and the formation of granulation tissue and matrix formation are reduced (Miyazaki et al., 1995; Noiri et al., 1999; Chabas et al., 2001; O'Regan et al., 2001; Yumoto et al., 2002). OPN was also shown to regulate cell survival and protection of cell survival in endothelial, immune, and various tumor cells (Scatena et al., 1998; Khan et al., 2002; Rittling et al., 2002). Interestingly, a lack of OPN expression in post-myocardial infarction and cardiac fibroblasts is associated with reduced programmed cell death (PCD) as detected by TUNEL reagent and an increase in rapid necrotic cell death in cardiac fibroblasts (Zohar et al., 2004).

Expression of OPN in the normal and inflamed intestinal mucosal tissues (Qu and Dvorak, 1997; Gassler et al., 2002) has indicated a role for OPN in protecting the intestine from pathogens and in the development of inflammatory bowel diseases (IBD) (Masuda et al., 2003). However, despite the potential importance of OPN expression in gut tissues, little is known of the role of OPN in the normal or diseased intestine. To investigate the role of OPN in IBD, we have used dextran sulfate sodium (DSS) to induce experimental colitis in the colon of mice (Okayasu et al., 1990) with a targeted disruption of the OPN gene. The DSS model exhibits inflammatory and histopathological changes that are characteristic of colitis in humans (Okayasu et al., 1990; Mahler et al., 1998; Steidler et al., 2000; Sasaki et al., 2003), and has been used extensively to study various aspect of acute and chronic colitis. This model is also used to analyze the patency and repair of the epithelial barrier (Diaz-Granados et al., 2000), leukocyte behavior, lymphocyte responses, and the release of pro-inflammatory cytokines (Dieleman et al., 1998; Rachmilewitz et al., 2002; Naito et al., 2004). The inflammatory reaction appears
to be induced by phagocytosis of DSS particles by macrophages (Okayasu et al., 1990; Stevceva et al., 2001) and upon withdrawal of DSS, the destructive effects are generally reversible with the regeneration of colon mucosal tissues. Using this model to compare changes in the pathophysiological responses associated with the lack of OPN expression, we have found that changes in immune cell function are associated with decreased TNF-α expression and, in contrast to other inflammatory diseases in OPN-null mice (Miyazaki et al., 1995; Noiri et al., 1999; Chabas et al., 2001; O'Regan et al., 2001; Yumoto et al., 2002), there is an exacerbation of disease resulting in massive destruction of intestinal crypts. These studies, therefore, demonstrate the importance of OPN in mucosal protection.

**MATERIALS AND METHODS**

**Experimental acute colitis**

The generation and initial characterization of OPN-null mice has been described previously (Rittling et al., 1998). The original 129sv F2 mice were subsequently backcrossed (10×) into a C57BL/6J background. Adult, 8 to 10-week-old OPN-null mice and their matched C57BL/6J wild-type (WT) controls were fed DSS (DSS - mol wt. 36,000-50,000; MP Biomedical, Inc., Aurora, OH) 5% (wt/vol) dissolved in sterile distilled water for 7 days to induce experimental colitis (Okayasu et al., 1990). Control animals were given water only and the experimental and the control (sham) animals examined for water consumption. Animals were housed in a specific pathogen-free facility with controlled temperature and light/dark cycles, and fed standard mouse chow pellets. Mice
were observed on daily basis for 7 days and examined for the clinical progression of colitis. Animal experiments were conducted according to guidelines established by the Animal Care Committee of the University of Toronto.

**Clinical assessment of colitis**

*Body weight and disease activity index (DAI)*

Body weight, stool consistency, and anal bleeding were monitored daily. The disease activity score was assessed using a disease activity index, essentially as described by Hartmann et al. (2000) with minor modifications. Each score was determined as follows: *0* was given for well formed pellets and no anal bleeding, *1* for pasty and semi formed stools that did not stick to the anus and/or rectal dot bleeding, *2* pasty stool and bleeding, and *3* for liquid stool that remained adhesive to the anus and/or for perfuse bleeding (blood observed on the cage wall). Scores were added and then divided by the number of animals in each group to calculate the mean and standard error of means.

*Peripheral blood cell analyses*

Approximately 0.5-1 ml of blood was collected by intra-cardiac puncture in labeled vials containing 1,000 µ/ml heparin and sent on ice for hematological analysis to VITA-TECH Laboratories (Toronto, Ontario). Blood samples were analyzed for total blood and differential white blood cell (WBC) counts, hemoglobin, hematocrit, mean
corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and MCH concentration (MCHC).

**Colon length and spleen weight**

Animals were killed after 3 or 7 days of treatment, colons were carefully dissected and their length was measured from the ileocecal junction to the anal verge, as changes in length have been shown to be associated with progression of colitis in this model (Okayasu et al., 1990). Spleens were also removed aseptically and weighed, since enlargement of the spleen is characteristic of a systemic inflammatory reaction as occurs in acute DSS-colitis, where it has been correlated with disease severity (Axelsson et al., 1998; Morteau et al., 2000).

**Histopathology**

Colon sections were prepared from an area extending from the rectum to 2 cm distal to the cecum. Notably, preliminary pathological analyses did not find any marked histological variation in the different areas of this colon section. Sections of the small intestine, which does not exhibit inflammatory changes in the DSS-treated animals (Okayasu et al., 1990), were also analyzed as an additional intra-animal control. Preparation of gut specimens was done using conventional procedures. Briefly, colons were washed with cold phosphate-buffered saline (PBS) fixed in 10% formalin, dehydrated in ethanol, and embedded in paraffin. Five-micrometer thick sections were stained with hematoxylin and eosin and analyzed by two blinded observers. Scoring of histological changes was done as previously described (Cooper et al., 1993) for this
model. The following grading scale was used for crypts changes: “0” intact crypt; “1” loss of the basal one-third of the crypt; “2” loss of the basal two-thirds of the crypt; “3” loss of the entire crypt with the surface epithelium remaining intact; “4” loss of both the entire crypt and surface epithelium (erosion). Scoring was quantitated for the grade and percentage area of involvement with the product of the two being the crypt score. The scores for each piece of tissue were summed and divided by the number of pieces. The maximum possible histological score was 400.

**Osteopontin and TUNEL immunohistochemistry**

The expression of OPN in mouse colons was examined by immunohistochemical analysis. Briefly, tissue sections were de-paraffinized, re-hydrated, autoclaved for 2 min at 120°C, then immersed in 10 mM sodium citrate (pH 6.0) for antigen retrieval, and incubated with 3% H2O2 for 30 min to eliminate endogenous peroxidase activity. After blocking with 5% normal goat serum and then incubating with in 0.5% BSA in PBS, pH 7.4, for 30 min, the sections were incubated overnight with a rabbit anti-porcine OPN antibody, (Zhang et al., 1990) affinity-purified using recombinant rat OPN (provided by Dr. H.A. Goldberg, University of Western Ontario, Ontario, Canada) and sections were incubated with biotinylated goat anti-rabbit IgG for 30 min. Notably, we have found this antibody to be the most specific of several antibodies tested for mouse OPN. PCD was detected in sections using Apo-BrdU-IHC™ In Situ DNA Fragmentation Assay Kit (BioVision, Inc., Mountain View, CA) according to the supplier’s protocol. Quantitation of TUNEL positive nuclei was performed in sections without counterstaining and the staining intensity was determined using image analyses (ImajJ software; NIH, Bethesda,
MD). Immunoreactivity of OPN staining and TUNEL was detected by the avidin-biotin-peroxidase complex method (Vectastain ABC kit; Vector Laboratories, Burlington, Canada) and stained sections photographed on a Nikon Eclipse E400 microscope.

**Myeloperoxidase activity and gelatin enzymography**

Myeloperoxidase activity was analyzed in gut tissue samples to evaluate neutrophil accumulation using a commercial kit for the o-dianisidine assay (Cytostore, Calgary, Alberta, T2L 2K8, Canada). Briefly, weighed gut strips washed in PBS and stored at -80°C were thawed, homogenized in a 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0) and clarified by centrifugation for 2 min at 10,000 rpm in a microcentrifuge at 4°C. An aliquot of the supernatant was allowed to react with the chromogen (o-dianisidine dihydrochloride) in the presence of potassium phosphate buffer (monobasic potassium buffer, dibasic potassium phosphate buffer, and 1% H$_2$O$_2$). The absorbance was measured immediately by spectrophotometry at 450 nm and again 60 sec after the addition of the chromogen to determine the reaction rate. Murine bone marrow neutrophils, isolated on a Percoll gradient, served as a positive control. Myeloperoxidase activity was expressed as the quantity of enzyme in 100 mg tissue degrading 1 µmol/min of peroxide at 37°C.

Gelatin enzymography was used to detect and quantify precursor and activated forms of gelatinases including matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9). Aliquots from the supernatant used for myeloperoxidase activity were mixed with 25% 4× sample buffer and 20 µl samples subjected to electrophoresis on 10% SDS-PAGE gels
containing 160 µg/ml gelatin. After electrophoresis, the gels were washed in 2.5% Triton-X-100 to remove the SDS, then incubated for 24 h at room temperature (RT = 21°C) before staining with 0.5% Coomassie blue. The gels were photographed on a Nikon Eclipse E400 microscope and analyzed by densitometry.

**Splenocytes and gut tissue analyses**

To identify immune cell responses, spleens were fractionated and sieved to obtain single cell (splenocyte) preparations, splenocytes were counted using the Beckman Coulter Z1 (Burlington, ON, Canada) and then suspended in ice-cold ammonium chloride solution to lyse red blood cells. After pelleting by centrifugation, cells were re-suspended in RPMI 1640 medium supplemented with 5% fetal calf serum and 10% antibiotics for culturing, or were re-suspended in PBS for flow cytometry single cell analyses. Flow cytometry was performed using $3 \times 10^6$ splenocytes/sample. Cells were labeled with the following antibodies: phycoerythrin (PE) anti-mouse CD4 (L3T4, BD Biosciences, Pharmingen, Mississauga, Canada), fluorescein isothiocyanate (FITC) anti-mouse CD8a (Ly-2- BD Biosciences, Pharmingen) and PE tagged affinity-purified rat anti-mouse F4/80 (Serotec, Cedarlane Laboratories, Ontario, Canada). Stained cell suspensions were analyzed with a FACStar Plus flow cytometer (Becton Dickinson FACS Systems, Franklin Lakes, NJ).

To determine the expression of cytokines by splenocytes, cultured spleen cells were stimulated with the T-cell mitogen concanavalin A (Con A; 2.5 µg/ml) or LPS (10
ng/ml) for 48 h at 37°C. Cell supernatants and homogenized gut tissue samples (prepared as described above for the myeloperoxidase and gelatinase analyses) were collected and analyzed with an antibody-based protein array system to detect secreted pro-inflammatory cytokines using Tran-Signal™ mouse cytokine antibody arrays (Panomics, Fremont, CA). Relative expression of each cytokine was quantified by densitometry and calculated as a percentage of the positive and negative intra membrane controls.

**Statistical analyses**

Results are expressed as mean ± standard error of the means (mean ± SEM). Experiments in vivo were performed at least five times with three or more mice in each group, and analysis of tissues and cells in vitro performed at least three times. The data were compared using one-way ANOVA analysis with the Bonferroni method as post hoc test. Statistical significance was defined as $P < 0.05$ and calculations were performed using the statistical analysis software SPSS (SPSS, Inc., Chicago, IL).

**RESULTS**

**Augmentation of OPN expression in DSS-induced colitis**

Since OPN is constitutively expressed in the human intestinal mucosa, with increased expression associated with inflammatory bowel disease lesions (Gassler et al., 2002; Masuda et al., 2003; Sato et al., 2005), we first examined the pattern of OPN expression in the intestines of normal mice and mice with DSS-induced colitis by immunostaining histological sections. In control mice, OPN staining was evident in the
epithelial as well as mucosal layers of the colon (Fig. 3.1A), while no staining was observed in the absence of primary antibody (Fig. 3.1B). DSS treatment increased staining markedly for OPN in WT mouse (Fig. 3.1C) colons and, as expected, no staining was observed in OPN-null mouse colons (Fig. 3.1D). Positive staining was detected across the intestinal mucosa with the highest intensity seen in the inflamed epithelial layer.

**DSS-induced colitis in the absence of OPN results in aggravated inflammation**

Treatment of mice with DSS produced clinical and histological signs of colitis in WT as well as OPN-null mice. Notably, animals segregated according to gender showed no significant differences in body weight, disease activity index (DAI), peripheral blood cell analyses, colon shortening, spleen weights, or histology. OPN-null and WT mice responded to DSS administration with ~25% overall weight loss over the 7 days with no significant difference between the groups other than on Days 5 and 7 at which time DSS-treated WT mice exhibited <5% more weight loss than the DSS OPN-null mice (Fig. 3.2A; $P < 0.05$). Signs of clinical disease, as determined by the DAI, were usually apparent at Day 3 with a 12-24 h earlier onset for WT mice (Fig. 3.2B). OPN-null mice treated with DSS also exhibited a higher DAI after Day 4, which was significantly higher (25-70%; $P < 0.05$) than the DSS-treated WT mice.
**Figure 3.1.** Immunostaining for OPN in normal and diseased mouse colons. 

A: Immunostaining of WT colon shows OPN to be constitutively expressed by epithelial cells of the crypts as well as some staining in submucosal layers where endothelial, immune, and fibroblasts expressing OPN can be identified. 

B: Control staining of WT colon in the absence of primary antibody. 

C: Immunostaining of DSS-treated WT colon showing increased OPN expression by most of the crypt epithelial cells and high intensity staining in cells of the sub-mucosal layers, including the inner muscularis layer, the external muscularis layer and epithelial cell of the mesothelium. 

D: Immunostaining of DSS-treated OPN-null mouse colon, which exhibits increased destruction of the normal gut architecture and massive infiltration of immune cells, shows no immunoreactivity for OPN.
Colon shortening, used as a morphometric measure for the degree of inflammation, was >30% in DSS fed mice ($P < 0.001$), with colons being significantly shorter ($P < 0.001$) in the DSS-treated OPN-null mice ($P < 0.001$) than the DSS-treated WT mice (Fig. 3.2C). Spleen enlargement, as a marker of severity of DSS-induced colitis, was significantly ($P < 0.001$) increased >70% in DSS-fed mice and to greater degree (>100%; $P < 0.004$) in DSS-treated OPN-null mice compared to WT mice (Fig. 3.2D). Thus, the spleen enlargement reflected the degree of disease activity in response to treatment, which caused more severe bleeding and anemia in the DSS-treated OPN-null mice. However, despite the spleen enlargement there was no significant increase in spleen cell numbers (WT sham- $5.27 \times 10^6$/ml, OPN-/- sham- $4.64 \times 10^6$/ml, WT DSS- $5.1 \times 10^6$/ml, OPN-/- DSS- $5.67 \times 10^6$/ml; RBC were lysed prior to counting $P > 0.05$). Notably, colons and spleens examined on Day 3, showed no apparent differences between the DSS-treated animals and their sham controls (data not shown), consistent with the absence of clinical signs evaluated by the DAI scores (Fig. 3.2B).
**Figure 3.2.** Clinical progression of DSS-induced colitis. **A:** DSS-treated mice (solid bars) exhibited a weight loss <25% of their original weight by Day 7 (\( P < 0.001; ** \)). DSS-treated WT mice had a significantly greater loss than the DSS-treated OPN-null mice only on Day 5 (\( P < 0.05; * \)). **B:** DSS-treated OPN-null mice exhibited 20-50% higher scores of disease activity index (DAI) after the 4th day of DSS administration, being significantly higher than DSS-WT mice on Days 5 and 7 (\( P < 0.05; * \)). **C:** Colon length, as a morphometric measure for the degree of colitis in DSS-treated animals, was shortened ~40% of the control (sham) non-treated mice. Colon shortening was significantly greater for the DSS-OPN-null mice (\( P < 0.001; ** \)). **D:** Spleen enlargement, as a marker of systemic inflammation, was calculated in proportion to the body weight. A twofold increase in the spleens of DSS-treated WT mice was exceeded by a 2.5-fold increase in the DSS-treated OPN-null mice (\( P < 0.05; * \)).
Heparinized peripheral blood collected by intra-cardiac puncture on Days 3 and 7 revealed decreased values for both hemoglobin and the hematocrit in all DSS-treated animals on Day 7, indicative of hematochezia and blood loss (data not shown). This was also reflected in the decrease in red blood cells (RBCs) counts of the DSS-treated mice on Day 7 (Fig. 3.3A; black bars). However, no significant differences were evident between OPN-/- and WT mice in any of these parameters ($P > 0.2$); White blood cell counts (WBCs) in the blood of OPN-null mice treated with DSS were $\sim$2.5-fold higher than in DSS-treated WT mice ($P < 0.001$) on Day 3, whereas a similar difference was observed for the WBC in the DSS-treated WT ($P < 0.005$) on Day 7, at which time the WBCs had decreased in the OPN-null mice (Fig. 3.3A,B). Differential counts of the WBC showed that the increase in the DSS-treated OPN-null mice on Day 3 could be attributed to a 2.5-fold increase in lymphocyte number (Fig. 3.3C; $P < 0.001$) with no significant differences in the numbers of bands, monocytes, eosinophils, and basophils (data not shown), while neutrophil numbers were elevated modestly in both WT and OPN-null mice. On Day 7, increases in WBC counts in the WT mice treated with DSS translated into a $\sim$20-fold increase in neutrophil counts (Fig. 3.3F) as well as a $\sim$threefold increase in lymphocyte counts (Fig. 3D; $P < 0.001$). In DSS-treated OPN-null mice, the increase in lymphocytes was minimal, almost back to control levels on Day 7, while the neutrophil number was increased $\sim$fourfold (Fig. 3.3D,F; $P < 0.005$). Notably, despite the significantly higher disease state (DAI; Fig. 3.2B), the neutrophil numbers in the blood of DSS-treated OPN-null mice were lower than in the DSS-treated WT mice, which may reflect the constitutively lower neutrophil numbers in untreated OPN-null mice (Fig. 3.3E,F).
Figure 3.3. Peripheral blood analysis of DSS-induced colitis. A: On Day 3, blood samples showed an early increase in WBC counts for the DSS-treated OPN-null mice ($P < 0.001;**$), whereas WBC cell counts for the DSS-treated WT mice were unchanged. B: The WBC counts were significantly increased in DSS-treated WT mice on Day 7 ($P < 0.05;*$), while WBC counts had decreased and were not significantly greater than controls ($P < 0.02$). RBC counts were reduced for DSS-treated WT and OPN-/- mice on Day 7. C,D: Differential analyses of the WBCs showed that the increases of WBCs on Day 3 (C) and Day 7 (D) in DSS-treated mice were due to increased lymphocyte numbers. E: Neutrophils were increased on Day 3 in DSS-treated mice but not significantly. F: On Day 7, neutrophils increased 20-fold ($P < 0.001;**$) in DSS-treated WT mice with a significantly lower ($P < 0.001;**$) fourfold increase in the DSS-OPN-/- mice.
Total crypt destruction in the absence of OPN

Analysis of histological scoring for destruction in these studies was limited to the epithelial layer (Cooper et al., 1993). Histological examination of colon sections from control mice showed no inherent anatomical differences between colons from WT and OPN-null mice and no signs of inflammation were evident in either group of control mice (Fig. 3.4A,B). In the DSS-treated WT colons an inflammatory infiltrate, shortening of the crypt and loss of basal one-third of the crypt architecture was observed (Fig. 3.4C). In comparison, the DSS-treated OPN-null colons exhibited a dense inflammatory infiltrate and an overall crypt destruction in which the base of the crypts were frequently detached from the muscularis mucosae (Fig. 3.4D). The destruction of the crypts, measured by the crypt score, was significantly higher in the OPN-null DSS-treated mice (Fig. 3.4E; $P < 0.001$), indicating exacerbated tissue destruction in the absence of OPN expression. In contrast, there were no significant signs of inflammation or destruction in the small intestine of DSS-treated mice (not shown).

Increased colon destruction in OPN-null mice is associated with high MPO activity of colon infiltrating neutrophils

Myeloperoxidase activity (MPO), reflecting neutrophil activity, was increased markedly in samples of colon tissue from DSS-treated mice, with a higher increase clearly evident in the colons of OPN-null mice (Fig. 3.5A; $P < 0.043$), consistent with greater tissue destruction in OPN-null mouse colons (Fig. 3.4E). Gelatin enzyunography revealed precursor and activated forms of matrix metalloproteinase-2 (MMP-2) in the
Figure 3.4. DSS-induced histological changes in the colon. Photomicrographs of hematoxylin and eosin-stained colon sections. A, B: No differences in histological features were evident between the sham OPN-null and WT colons. C: In DSS-treated WT mouse colons an inflamed infiltrate was observed with shortening and loss of the basal one-third of the crypt. D: In DSS-treated OPN-null colons a total loss of crypt structure was seen with a dense coverage of the inflammatory infiltrate. E: Destruction of the intestines analyzed by crypt score showed a >60% significantly ($P < 0.001$; **) higher destruction in the DSS-treated OPN-null mice compared to the DSS-treated WT mice.
DSS-treated animals, but MMP-9 could not be detected (Fig. 3.5B). While the MMP-2 was produced in higher quantities in the DSS-treated animals, there was no significant difference in quantities of the pro-enzyme between the DSS-treated WT and OPN-null colons. However, the activated MMP-2 was twofold lower in the colons from OPN-null mice.

**Lack of differentiation of inflammatory cells accompanied by reduced production of TNF-α**

Flow cytometry single cell analysis of cells from control spleens of WT and OPN-/- splenocytes showed that cells have similar size and volume characteristics (Fig. 3.6A). After 7 days of DSS treatment, a new subpopulation of larger cells (higher forward scatter) in both WT and OPN-null groups (arrow heads) appeared, albeit with a smaller number of this new sub-population of cells in the OPN-null spleens and many splenocytes exhibited the same forward and side scatter characteristics observed in the sham animals (green arrow). Staining of cells for major lymphocyte subsets (CD4+, CD8α+) and for differentiated macrophages (F4/80) showed a similar pattern in the controls, whereas two or more CD4+ and CD8α+ sub-populations (fluorescence intensity) appeared in the WT splenocytes following DSS treatment, together with a higher number of differentiated macrophages (Fig. 3.6B). In comparison, only one lymphocyte subpopulation was observed in the OPN-null splenocytes and there was a lower number of differentiated macrophages.
A

% Increase in MPO activity

-20
0
20
40
60
80
100
120
140

WT DSS small intestine
OPN-/-DSS small intestine
WT DSS colon
OPN-/-DSS colon

Small Intestine
Day 3
Day 7
Colon

B

Pro-MMP-9
Pro-MMP-2
Activated MMP-2

92 kDa
72 kDa
61 kDa
55 kDa

WT sham
OPN-/- sham
WT + DSS
OPN-/- + DSS
Figure 3.5. Biochemical analyses of myeloperoxidase activity and gelatin enzymography. A: Myeloperoxidase activity, as an indicator of neutrophil infiltration, in DSS-treated OPN-null mouse colons samples showed ~threefold greater activity compared to DSS-WT colon samples ($P < 0.001$). B: Enzymographic analyses of representative colon samples showed the presence of both the precursor and activated forms of matrix metalloproteinase-2 (MMP-2) in the DSS-treated animals, but slower migrating bands for MMP-9 could not be detected. The activated MMP-2 band was ~twofold lower in the DSS-treated OPN-null mice colons compared to DDSS-treated WT samples.
Splenocytes from Day 3 DSS-treated animals did not differ from the sham controls in their relative proportions of CD4+, CD8α+ lymphocytes or macrophages (data not shown). However, on Day 7, in spleens of OPN-null mice with DSS-induced colitis there was a significant reduction in the proportion of macrophages (WT, \(P < 0.047\); OPN-/−, \(P < 0.001\)), as well as CD8α+ (WT, \(P < 0.027\); OPN-/−, \(P < 0.018\)), and CD4+ lymphocytes (\(P < 0.02\)) (Fig. 3.6C). The overall decreases for the different splenocyte populations in DSS-treated OPN-null mice were significantly greater than in the DSS-treated WT mice (\(P < 0.05\)) in spite of the higher spleen enlargement in the DSS-treated OPN-null mice (Fig. 3.2D).

Cultured splenocytes stimulated with LPS for 48 h were analyzed for their production of pro-inflammatory cytokines. To emphasize the major changes values were calculated as a percentage of the positive controls (100%) on each array. In response to LPS, the cytokines G-CSF, RANTES, and MIP1α were increased in WT splenocytes and further increased for G-CSF and RANTES in response to DSS-treatment, which also markedly elevated TNF-α and IL-4. In contrast, only minor increases in RANTES, MIP1α, and VEGF, were observed in the OPN-/− splenocytes and with DSS treatment only small increases were seen in the RANTES, MIP1α, and G-CSF. Notably, Con-A stimulation showed similar differences in cytokine production between splenocytes from the sham and DSS-treated WT and OPN-null mice (data not shown). Lower, cytokine levels were also detected in extracts of gut tissues from the OPN-null mice in which the levels of TNF-α increased by only 3.12-fold compared to a 4.67-fold (\(P < 0.05\)) increase in the DSS-treated WT colons.
**Figure 3.6.** DSS effects on splenocytes. **A:** Flow cytometric analysis showed similar forward- and side-scatter characteristics for splenocytes from sham WT and OPN-null mice. Following DSS treatment, a new sub-population of splenocytes appeared (black arrow). Notably, this new subpopulation was smaller in the OPN-/- splenocytes, while many cells retained the morphological properties of the sham splenocytes (blue arrow) (>10^5 cells were analyzed). **B:** Splenocytes stained for CD4, CD8α (lymphocytes), and F4/F80 (differentiated macrophages) showed no differences between the sham splenocytes. In the DSS-stimulated splenocytes, an increase in fluorescence intensity was observed for the WT and OPN-/- splenocytes. However, DSS-treated WT splenocytes exhibited a higher fluorescence intensity for differentiated macrophages and revealed one (CD8α+) or two (CD4+) additional subpopulations of lymphocytes (>10^5 cells were analyzed). **C:** The relative percentage of the different cell types was evaluated and, whereas no differences were observed for the sham animals, the DSS-treated mice exhibited a reduced number of macrophages (WT, \(P < 0.047\); OPN-/- \(P < 0.001\)) and CD4+ (WT, \(P < 0.064\); OPN-/- \(P < 0.02\)) and CD8α+ (WT, \(P < 0.027\); OPN-/- \(P < 0.018\)) lymphocytes in their spleens, the decrease being significantly greater for OPN-null mice (\(P < 0.044\)) (>10^5 cells were analyzed).
Non-programmed cell death in the absence of OPN

Since control of cell survival has been implicated as an important factor for the pathogenesis of IBD (Ina et al., 1999; Wittig et al., 2000; Kruidenier et al., 2003; Mikami et al., 2003), we stained sections from control and DSS-treated colons for TUNEL and determined the amount of TUNEL-positive nuclei in sections without counterstaining (Fig. 3.7B). Immunopositive nuclei were detected using NIH Image J pixel reader counts and scoring was quantitated in slides from five animals for each group with four fields.

No significant differences in TUNEL-positive cells were evident between OPN-/- and WT control tissues, or the DSS-treated OPN-null mice ($P > 0.1$); all exhibited a small number of TUNEL-positive nuclei. However, TUNEL-positive nuclei counts for the DSS-treated WT colons counts were >sevenfold higher ($P < 0.001$) than all the other groups.
Figure 3.7. A: Splenocyte secretion of pro-inflammatory cytokines. Splenocytes were stimulated for 48 h with LPS or Con-A and cytokine array analysis showed similar changes. Results are shown for LPS-treated cells. Changes in cytokine secretion were evaluated for each sample in relation to the average density of the negative controls (0%) and the average density of the positive controls (100%) on each cytokine array membrane and the values expressed as percentage change (mean ± SD). Significant increases of three- to sevenfold in the DSS-treated WT splenocytes were seen for in G-CSF, RANTES, TNFα, IL-4, and IL-6, whereas little or no change was detected in cytokine secreted by the sham and OPN-/- splenocytes. B: TUNEL staining of mouse colons. Programmed cell death (apoptosis) was detected using TUNEL reagent in cross-sections of colon tissues, without counterstaining. Image analyses quantitation of staining intensity was performed as a reflection of TUNEL positive nuclei. No significant differences were evident in the number of TUNEL-positive nuclei between OPN-null control, WT control or the DSS-treated OPN-null mice ($P > 0.1$;**), whereas counts in the DSS-treated WT colons were sevenfold higher ($P < 0.001$;***) than in the control groups.
DISCUSSION

Previous studies have identified the significant contribution of OPN to the progression and sequelae of various inflammatory diseases in models of arthritis, kidney, and lung fibrosis and multiple sclerosis. (Miyazaki et al., 1995; Noiri et al., 1999; Chabas et al., 2001; Yumoto et al., 2002). In these pathologies, OPN expression by activated macrophages, T cells, and fibroblastic cells is increased as part of the inflammatory response and in the subsequent repair process. Consistent with this, we observed increased OPN expression with DSS treatment in WT mice (Fig. 3.1C), suggesting the importance of OPN in DSS-induced colitis. However, whereas previous studies have shown that inflammatory disease activity is attenuated in the absence of OPN, our studies show a greater susceptibility of OPN-null mice to DSS-induced colitis. Clinical parameters of weight loss, disease activity scores, colon shortening, and spleen enlargement were significantly greater in OPN-null mice and accompanied a decreased inflammatory cytokine response and an exacerbation of intestinal tissue destruction. In contrast to the detrimental effects of OPN associated with a protracted Th1 immune response in other inflammatory diseases (Miyazaki et al., 1995; Noiri et al., 1999; Chabas et al., 2001; O'Regan et al., 2001; Yumoto et al., 2002), here, we demonstrate the importance of the protective functions of OPN in colitis; a mucosal disease in which the production of pro-inflammatory cytokines, including TNF-α, and an intact mucosal epithelial barrier are required to combat a continuous bacterial influx.

The DSS model of colitis used in this study exhibits the clinical pathophysiology of acute colitis in mice with epithelial ulceration, crypt destruction, and inflammatory cell
infiltration of both the innate and the adaptive pathways. Since pro-inflammatory cytokines mediate both protective as well as destructive effects of inflammation, this model has been used to assess the effects of altered expression of inflammatory mediators such as: TNFα (Naito et al., 2003), TGFα (Egger et al., 1998), TGFβ1 (Beck et al., 2003), cyclooxygenase 1 and 2 isoforms (Morteau et al., 2000), nitric oxide synthase (Sasaki et al., 2003), IL-17 (Ogawa et al., 2004), IL-6 (Naito et al., 2004), and IL-10 (Steidler et al., 2000). The inflammatory response in this acute colitis model is the result of rapid recruitment and activity of macrophages and neutrophils, phagocytosing particles (Stevceva et al., 2001), and initiating lymphocyte proliferation and activity (Dieleman et al., 1998). In our studies, the activity of neutrophils, as demonstrated by myeloperoxidase activity, correlated with the amount of destruction. Notably, while previous studies have indicated that the differentiation and activity of monocytes and lymphocytes are impaired in the absence of OPN expression (McKee and Nanci, 1996; Giachelli et al., 1998; Ashkar et al., 2000; Chabas et al., 2001; Bourassa et al., 2004; Zhu et al., 2004), we have found that the cytocidal and phagocytic activities of OPN-/- neutrophils studied in vitro are normal (unpublished observations). Since neutrophil infiltration is an immediate response to mucosal injury that leads to the development of an acute inflammation, in the absence of OPN there is a persistence and increased destructive activity of neutrophils. This may be due to the slow clearance of apoptotic neutrophils by OPN-/- macrophages (Haslett et al., 1994; Hart et al., 2000), which display impaired migration and differentiation (Zhu et al., 2004) and may also have reduced phagocytic activity. Neutrophil hyperactivity is also associated with an increased oxidative burst, which increases cell death in IBD (Kruidenier et al., 2003) and may be accelerated as necrotic
cell death in the absence of OPN protection (Zohar et al., 2004). This interpretation is consistent with the increased tissue destruction (Fig. 3.1) and markedly elevated PMN myeloperoxidase activity (Fig. 3.4E) that we have observed in OPN-null mice, in which little epithelial regeneration occurs during remission.

Clearance of neutrophils and activation of the adaptive immune response is particularly important in protecting mucosal tissues, which are continuously challenged by pathogens and noxious agents. The adaptive immune response is triggered by a specific recognition between resident APC/dendritic cells and naïve T cells that results in the differentiation of Th1, T-helper, and cytotoxic (NKT) T cells, which control the immune reaction. In response to DSS, new subpopulations of CD4+ and CD8α+ lymphocytes and an increase in macrophages were seen in cytograms of spleen cells from DSS-treated WT mice. In contrast, only a single subpopulation of CD4+ and CD8α+ lymphocytes was observed together with lower numbers of macrophages, in DSS-treated OPN-null mice (Fig. 3.6B), consistent with a lack of lymphocyte and macrophage differentiation in OPN-null mice (McKee and Nanci, 1996; Giachelli et al., 1998; Ashkar et al., 2000; Chabas et al., 2001; Bourassa et al., 2004; Zhu et al., 2004). Although T-cell proliferation in the OPN-null mice is evident from the early increase of lymphocytes in peripheral blood on Day 3, the subsequent differentiation of T-cells into effector CD4+ and CD8α+ lymphocytes appears to be impaired. This finding also suggests that OPN deficient lymphocytes may remain in the peripheral blood, unable to enter the inflammatory site and are, therefore, not able to fulfill immune modulatory functions in the inflamed gut. Since OPN is thought to control APC/dendritic cell maturation and survival (Kawamura et al., 2005), and also promotes their emigration from the epidermis
to secondary lymphatic organs to elicit Th1 responses (Renkl et al., 2005), activation of the adaptive immune response and mobilization of lymphocytes from lymph nodes would be expected to be impaired in the absence of OPN. Thus, in combination with the impaired macrophage function, important immune defense mechanisms required for the resolution of the inflammatory response appear to be compromised in the absence of OPN expression resulting in the persistence of neutrophil activity (Fig. 3.5A) that causes extensive tissue destruction (Fig. 3.4E).

Secretion of pro-inflammatory cytokines is a pre-requisite for host defense, and immune response and current therapeutic approaches are directed towards the abrogation of inflammatory signaling by mediators such as TNF-α or IL-10 (Ashcroft et al., 1999; Beck et al., 2003; Myers et al., 2003; Palladino et al., 2003). Notably, contrasting increases in G-CSF, RANTES, IL-4, and MIP-1α together with <10-fold increase in TNF-α observed in WT mice treated with DSS, splenocytes from DSS-treated OPN-null mice did not exhibit significant increases in the production of pro-inflammatory mediators relative to their sham controls. The impaired cytokine response may be responsible for the reduced macrophage responses (Renkl et al., 2005) leading to neutrophil hyperactivity and a lack of TNF-α-regulated cell survival, resulting in the higher DAI scores in DSS-treated OPN-null mice.

The tissue destruction in the colitis lesions observed in response to DSS is accompanied by considerable cell death, particularly in the OPN-null mice. Quantitative analyses of cell counts (excluding immune cells) in H&E slides of colons demonstrated a significant threefold reduction (data not shown) in the cellularity in the mucosa of DSS-
treated OPN-null colons, compared to both the sham animals and the DSS-WT colons. However, whereas our analyses of TUNEL-positive cells (Fig. 3.7B) showed apoptotic cell death in WT mice, the destruction and reduced cellularity observed in the OPN-null mice is not the result of PCD. Notably, while required for cell survival (Rogers et al., 1997; Scatena et al., 1998; Weintraub et al., 2000; Khan et al., 2002; Zohar et al., 2004), OPN also supports PCD, which prevents uncontrolled rapid necrotic death (Zohar et al., 2004) that can intensify inflammatory destruction and loss of epithelial barrier protection (James, 1998; Nicotera et al., 1999; Naito et al., 2003). In contrast to PCD, in which dying cells go through a process of controlled intracellular enzymatic degradation and are phagocytosed to clear the way for new cells to repair the matrix, necrotic cell death is strongly associated with the destruction of cell membranes and the expulsion of noxious intracellular contents that exacerbate inflammation and increase of tissue destruction. Thus, the disintegration of the colon epithelial layer, followed by massive destruction of the colon mucosa in the absence of OPN (Fig. 3.4D) provides definitive evidence of the importance of OPN in mucosal barrier defense.

Expression and secretion of TNF-α can initiate PCD through activation of TNF-α receptors (TNFR), and expression of the Fas-associated death domain (FADD) and the TNF-related apoptosis inducing ligand (TRAIL). Notably, TNF-α signaling is also important for the immune and inflammatory responses as well as promoting cell survival through the same receptors (i.e., TNFR1) that can also initiate cell death (Ruemmele et al., 1999; Liu, 2005). In this respect, the ability of OPN to support inflammation in arthritis, renal and lung diseases, while having contrasting effects on colitis in OPN-null mice is suggestive of similarities in the biological activities of OPN and TNF-α. Thus,
TNF-α can promote cell survival, inflammation, and matrix remodeling (activation of pro-MMP2) through activation of NF-κB pathways (Muppidi et al., 2004). In similar manner, OPN promotes endothelial cell survival through NF-κB activation (Scatena et al., 1998) and pro MMP-2 activation in tumor cells (Philip et al., 2001). Further, the pattern of destruction in DSS-induced experimental colitis in OPN-null mice and the identification of neutrophils as the destructive cells is similar to that observed in TNF-α null mice (Naito et al., 2003), in which there is also an exacerbated destruction of the epithelial barrier due to persistent neutrophil hyperactivity. Interestingly, part of the destructive ability of TNF-α is attributed to nitric oxide production, which increases the expression of OPN (Guo et al., 2005), which acts as a negative regulator for this pro-inflammatory agent (Rollo et al., 1996). Moreover, ablation of endothelial nitric oxide synthase expression and nitric oxide production in the murine DSS model results in aggressive tissue destruction, which is primarily the result of neutrophil activity (Sasaki et al., 2003). Thus, the lack of OPN expression in our studies can be associated with diminished functionality of cytokines such as TNF-α that results in an un-controlled immune response augmented by rapid cell necrosis leading to massive tissue destruction.

In addition to an impaired immune response, fibroblast function also appears to be compromised in the DSS-treated OPN-null mice, consistent with the impaired wound healing as reported previously (Liaw et al., 1998; Jander et al., 2002; Yumoto et al., 2002). In wound healing, matrix remodeling is necessary for the formation and subsequent resolution of scar tissue. Matrix metalloproteinases (MMPs) derived from macrophages, neutrophils, and from activated fibroblasts (Vaalamo et al., 1998; Pirila et al., 2003), augment matrix remodeling and promote overall tissue destruction. The
absence of MMP-9 in colitis gut tissues (Fig. 3.5B) indicates that the gelatinase activity is not from neutrophils and may be derived from macrophages or mesenchymal cells, which are known to express MMP-2. Moreover, the greater activation of MMP-2 in the DSS-treated WT animals may be associated with tissue repair, given the selective increase in the expression of this MMP by the profibrotic cytokine TGF-β (Overall et al., 1991). Alternatively, the difference may be the result of less activation of MMP-2 or decreased cellularity that is evident in the looser mucosa of DSS-treated OPN-/- colons. Whereas the matrix-promoting and immunoregulatory activity of TGF-β1 has been studied quite extensively (Wang and Mosmann, 2001; Kitani et al., 2003; Wahl and Chen, 2003), the role of OPN and its relation to TGF-β1-mediated activities are unknown.

In summary, these studies demonstrate the importance of OPN in maintaining the epithelial barrier, as well as ensuring a normal inflammatory response, to protect against DSS-induced colitis. The inability of OPN-null mice to retain the patency of the epithelial barrier reflects a dysregulated immune cell response involving deficiencies in pro-inflammatory cytokine production, which are likely to impact on cell survival and the progression of mucosal diseases.
Chapter 4: Role of Osteopontin in Neutrophil Function

ABSTRACT

Osteopontin (OPN) is important for the function of fibroblasts, macrophages and lymphocytes during inflammation and wound healing. In recent studies of experimental colitis we demonstrated exacerbated tissue destruction in OPN-null mice, associated with reduced tumour necrosis factor-α expression and increased myeloperoxidase activity. The objective of this investigation therefore was to determine the importance of OPN expression in neutrophil function. Although, in contrast to macrophages, neutrophils expressed low levels of OPN with little or no association with the CD44 receptor, intraperitoneal recruitment of neutrophils in OPN-null mice was impaired in response to sodium periodate. The importance of exogenous OPN for neutrophil recruitment was demonstrated by a robust increase in peritoneal infiltration of PMNs in response to injections of native or recombinant OPN. In vitro, OPN−/− neutrophils exhibited reduced chemokinesis and chemotaxis towards N-formyl methionyl leucyl phenylalanine (fMLP), reflecting a reduction in migration speed and polarization. Exogenous OPN, which was chemotactic for the neutrophils, rescued the defects in polarization and migration speed of the OPN−/− neutrophils. In contrast, the defensive and cytotoxic activities of OPN−/− neutrophils, measured by assays for phagocytosis, generation of reactive oxygen species, cytokine production and matrix metalloproteinase-9, were not impaired. These studies demonstrate that, while exogenous OPN may be important for the recruitment and migration of neutrophils, expression of OPN by neutrophils is not required for their destructive capabilities.
INTRODUCTION

Osteopontin (OPN) is a multifunctional cytokine that is up-regulated in a broad range of inflammatory diseases involving the brain, liver, gastrointestinal tract, lung, bone, cardiac tissue, joints and kidney (Giachelli and Steitz, 2000; O’Reagan and Berman, 2000; Denhardt et al, 2001a; Sodek et al, 2006). In recent studies we observed an exacerbation of intestinal tissue destruction in acute colitis induced in OPN-null mice (Da Silva et al, 2006). These findings contrast with the attenuation of experimental inflammatory disease observed in non-luminal tissues in OPN-null mice (O’Reagan et al, 2001; Miyazaki et al., 1995; Noiri et al., 1999; Chabas et al., 2001; Yumoto et al., 2002; Jansson et al, 2002), and emphasize the importance of OPN in mucosal protection (Sodek et al, 2006). Notably, OPN expression during inflammation is not limited to specific cell lineages but involves many different cells, including epithelial, mesenchymal, as well as immune, cells (Denhardt et al, 2001). The increased expression of OPN is reflected in elevated concentrations of OPN in tissue fluids and plasma and has been associated with increased cell mobilization, survival and activity. Many of the effects of OPN on macrophages and fibroblasts, which have a central role in regulating inflammatory and fibrotic responses, involve interactions with integrins and the CD44 receptor and are mediated by the cytoskeleton (Sodek et al, 2000).

Polymorphonuclear leucocytes (neutrophils, PMNs) and macrophages are rapidly activated to migrate to sites of infection in response to inflammatory signals. Migration of these cells is dependent upon cytoskeletal rearrangements that allow the cells to adopt a polarized morphology and directional movement in response to chemotactic stimuli.
Neutrophils are primarily responsible for the elimination of endogenous and exogenous noxious stimuli using a combination of phagocytosis, respiratory burst and release of proteases and cytotoxic mediators as the first line of host defence. Respiratory burst agonists include lipopolysaccharides (LPS), N-formyl methionyl leucyl phenylalanine (fMLP), and phorbol 12-myristate 13-acetate (PMA), which also stimulate the release of proinflammatory cytokines and phagocytosis. Under inflammatory conditions, rolling neutrophils attach to endothelial cells by reverse binding to transmembrane cell surface glycoprotein receptors. Although activated neutrophils migrating through the endothelial lining can cause tissue damage in facilitating access of immune cells to diseased sites, their presence is normally transient, allowing tissue remodelling to repair the damaged tissues. However, the persistent presence of hyperactivated neutrophils can cause irreparable tissue damage that impacts on organ function, as observed in colitis and other inflammatory diseases.

Previous studies have shown that OPN has a pivotal role in the development and maintenance of immune responses through its regulation of macrophage, lymphocyte and fibroblast activities. When challenged, all of these cells express high levels of OPN and exhibit impaired migration and cytokine production in the absence of OPN expression (Sodek et al, 2006; Miyazaki et al, 1995; Jansson et al, 2002; Diao et al, 2004; Sato et al, 2005; Ashkar and Weber, 2000; Matsui et al, 2003). OPN regulates cytokine expression by macrophages through interactions with cell surface integrins and CD44 receptors (Ashkar and Weber, 2000) while extracellular and intracellular interactions between OPN and CD44 have been implicated in the formation of cell processes and macrophage migration (Weber and Ashkar, 2000; Zhu et al, 2004). However, whereas CD44 is also
required for the polarization and directed migration of neutrophils, which migrate more rapidly than macrophages, the expression and localization of CD44 is largely independent of OPN (Sodek et al, 2006), suggesting that OPN may function differently in neutrophils.

We have observed an exacerbation of intestinal tissue destruction in OPN-null mice in response to colitis, which correlated with a suppressed tumour necrosis factor (TNF-α) response and increased myeloperoxidase activity (Da Silva et al, 2006), indicative of increased neutrophil activity. Notably, a similar pattern of destruction caused by the persistence of neutrophils has been observed in experimental colitis induced in TNF-α null mice (Naito et al, 2003). Thus, the objective of this investigation was to study the relationship between OPN expression and neutrophil function. Whereas recruitment of neutrophils was found to be impaired in OPN-null mice, the absence of OPN expression showed only modest effects on the polarization and directed migration of neutrophils and did not influence their destructive potential.

MATERIALS AND METHODS

Animals and cell preparations

Generation of the OPN-null mice has been described previously (Rittling et al, 1998). The original 129sv F2 mice were subsequently back-crossed (10 times) into a C57BL/6 J background. Adult, 8-week-old OPN-null mice and their matched C57BL/6 J wild-type (WT) controls were used for these experiments. Animal experiments were conducted according to guidelines established by the Animal Care Committee of the University of Toronto. Femurs and tibias were dissected and after flushing out the bone
marrow cells the neutrophils were isolated on an 82%/65%/55% Percoll gradient (Sigma, Oakville, ON, Canada). Following Wright–Giemsa staining (Sun et al, 2004), the cell preparations were shown to consistently contain > 90% neutrophils.

**Analysis of OPN expression by reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from WT and OPN−/− neutrophils and from RAW 264.7 macrophages using the Absolutely RNA miniprep kit (Stratagene, Sedar Creek, TX) and the purity was assessed spectrophotometrically. First-strand cDNA reactions were performed on 1 μg RNA in 20 μl 1 × PCR buffer II, containing 5 mM MgCl₂, 1 mM each of the dNTPs, 1 U/μl RNase inhibitor, 2.5 U/μl murine leukaemia virus (MuLV) reverse transcriptase, 2.5 μM random hexamers (Applied Biosystems, Foster City, CA) at 42° for 60 min. The cDNA (2.5 μl) was then amplified by PCR using Eppendorf MasterMix (Eppendorf North America Inc., New York, NY) in a 25 μl reaction volume in a GeneAmp PCR system 2400 (Perkin Elmer; Life Sciences, Boston, MA) for 35 cycles after an initial 30-second denaturation at 94°, annealing for 30 seconds at 55° and extending for 30 seconds at 72°. The sequences of the primers used for the amplification of murine OPN were: 5'-AGCCAAGCTATCACCTCGG-3' and 5'-GGTTTGCAGTCTTCTGCGGC-3’, which generated an amplicon of 408 base pairs (bp). The PCR products were resolved by electrophoresis on a 2% Nuseive gel and visualized by ethidium bromide staining. Quantitative real-time PCR was performed using Taqman PCR master mix and the ABI 7900 HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), and primer sets from ABI. The amplifications were
performed as follows: 2 min at 50°, 10 min at 95°, then 95° for 15 seconds and 60° for
60 seconds. The results were normalized to an internal control transcript encoding
glyceraldehyde 3-phosphate dehydrogenase (GAPDH). No PCR products were generated
from RNA before reverse transcription, indicating the absence of genomic DNA.

**Immunofluorescence staining and confocal microscopy**

Neutrophils were plated in eight-well chamber slides (Laboratory-Tek™; BD
Falcon, Bedford, MA) supplemented with α-minimum essential medium (α-MEM)
containing 5% fetal bovine serum and antibiotics (100 μg/ml penicillin G, 50 μg/ml
gentamicin sulphate and 0.3 μg/ml fungizone). Neutrophils were allowed to attach,
washed gently before fixing with paraformaldehyde [2% in phosphate-buffered saline
(PBS), pH 7.4 for 1 hr], and then stained with a rabbit anti-porcine OPN antibody (Zhu et
al, 2004) and a biotin-conjugated rat anti-mouse CD44 (Clone KM201, Cedarlane,
Laboratories, Hornby, ONT, Canada), followed by Texas red fluor-tagged goat anti-
rabbit F(ab’)2 fragment and fluorescein isothiocyanate (FITC) –streptavidin, respectively.
Other cells were stained with tetramethylrhodamine isothiocyanate (TRITC) -phalloidin
(Sigma Co., St Louis, MO). The immunostained cells were examined by laser confocal
microscopy as described previously (Zhu et al, 2004).

**Migration assays**

*Transwell chamber assays.*

For each group of mice, a neutrophil suspension (1 × 10^6/ml PBS) was plated
onto fetal bovine serum-coated 3.0-μm pore polycarbonate membrane inserts in two wells
of a 24-well Transwell plate (Corning Incorporated Life Sciences, Acton, MA), with $10^{-6}$ M fMLP (or PBS control) in the lower compartment as chemoattractant. Migration assays were also conducted to examine the effects of native OPN purified from RAW 264.7 cells on the migration when chemoattractants were included in the upper as well as the lower compartments. Neutrophils were allowed to migrate for 30 min at 37° through the membrane and attach to round, glass coverslips (12 mm diameter) placed at the bottom of the well. The number of attached cells on each coverslip was counted in 10 fields for each of the three replicate wells.

**Zigmond chamber assays.**

Bone marrow neutrophils were suspended in PBS and 1% gelatin for Zigmond chamber chemotaxis analyses using $10^{-6}$ M fMLP as the chemoattractant; analyses were performed as described previously (Sun et al, 2004; Alstergren et al, 2004). To quantify the proportion of cells that were polarized, the total cells and the number of cells that were polarized in any direction or towards the chemotactic gradient (< 45° from the direction to the gradient) were counted in four areas of images using time-lapse video microscopy (Nikon Eclipse E400) equipped with differential interference contrast optics and a × 40 objective. Images were captured at 60-second intervals and analysed with RETRAC software (http://mc11.mcri.ac.uk/) to characterize neutrophil chemotaxis. The data were expressed as means ± SD for each time-point.
**In vivo migration.**

Peritoneal cell recruitment was induced in three groups of three 8-week-old mice per group with a single injection of PBS containing either: 20 μg native rodent macrophage OPN purified to homogeneity from RAW 264.7 cell-conditioned medium, 20 μg full-length recombinant rat OPN, 5 mM sodium periodate (Sigma, Oakville, ON, Canada) or PBS alone (control). After 3.5 hr the animals were killed and 5 ml PBS was used as a lavage of the peritoneal cavity to collect the peritoneal cell infiltrate. The total cell number was counted and the relative percentage of neutrophils or mononuclear cells was determined by Wright–Giemsa staining.

**Phagocytosis assays**

General and specific phagocytosis by neutrophils stimulated with LPS (10 μg/ml in PBS) for 1 hr at 37° was assessed. For phagocytosis of polystyrene beads, Nile red (535/575) 1-0-μm carboxylate modified microspheres (Cat. No. T-8819; Molecular Probes, Eugene, OR) were opsonized with fetal calf serum, mixed with neutrophils at a ratio of 10 : 1 and incubated for 25 min at 37° in PBS. For measurement of phagocytosis through Fcγ or complement C5 receptors, sheep erythrocytes (sRBCs; ICN/Cappel, Aurora, OH) were washed with PBS and then opsonized with rabbit immunoglobulin G (IgG: ICN/Cappel; 1 : 2500) for 1 hr at 37°. Alexa 488-labelled monoclonal goat anti-rabbit secondary antibodies (Molecular Probes; 1 : 500) were added to label the sRBCs. For the complement pathway the sRBCs were opsonized with rabbit IgG (ICN/Cappel; 1 : 2500), and incubated with complement C5-deficient human serum (Sigma) for 30 min at 37°. Opsonized sRBC were labelled with Texas Red–sulphonyl chloride dye.
(Molecular Probes; 1 : 5) for 20 min at 4° and rinsed well. The labelled sRBCs were mixed with neutrophils at a ratio of 5 : 1 and incubated for 25 min at 37°; neutrophils were washed to remove non-phagocytosed RBCs before analyses. Phagocytosis was determined by flow cytometric analysis (Guava Personal Cell Analysis System; Guava Technologies Inc., Hayward, CA). The mean fluorescence index of > 10⁴ cells for triplicate samples was evaluated and the results were further confirmed by fluorescence microscopy (Nikon Eclipse CF160). The number of neutrophils with ingested erythrocytes/beads was counted for 100 neutrophils from each of the samples evaluated by flow cytometry.

**Measurement of neutrophil NADPH oxidase activity**

Neutrophil H₂O₂ production was assessed using the conversion of non-fluorescent dihydrorhodamine-123 reagent (DHR; Molecular Probes, Inc.) into rhodamine-123. Cells were incubated at 37° for 15 min with dihydrorhodamine-123 in the presence of fMLP or LPS; then, the mean fluorescence intensity of rhodamine 123 was detected by flow cytometry on a single-cell basis using a 490-nm filter; it was also confirmed by microscopy, as described above.

**Cytokine analysis and gelatin enzymography**

Suspended neutrophils (3 × 10⁶ cells/ml PBS) were incubated with 0.1 μM PMA at 37° for 15 min and centrifuged for 5 min at 16 000 g. Samples of the supernatant (500 μl) were mixed with an equal volume of assay buffer and incubated for 1 hr at 21°
with a murine cytokine array blot (TranSignal Mouse Cytokine Antibody Array 1·0; Panomics Inc., Fremont, CA). They were developed using chemiluminescence according to the manufacturer's instructions. Samples of supernatant (20 μl) and an equivalent sample of the cell pellets made soluble with lysis buffer were also electrophoresed on a non-reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (10% acrylamide and 0.1% gelatin) to analyse for gelatinase activity. The gels were washed twice for 10 min in 2.5% Triton X-100 to remove SDS and incubated in assay buffer (50 mM Tris–HCl, 0.2 M NaCl, 5 mM CaCl₂, 0.5 μl/ml Brij35) for 24 hr at 21° before staining with 0.5% Coomassie brilliant blue solution.

Statistical analyses

The results were expressed as means ± SD unless stated otherwise. For all experiments, at least three replicates were included and experiments were repeated at least three times. For multiple comparisons, analysis of variance (ANOVA) was performed.

RESULTS

Expression of OPN in neutrophils

The expression of OPN mRNA in neutrophils was measured by semi-quantitative RT-PCR and real-time quantitative (q) RT-PCR. Much lower levels of OPN cDNA were amplified from the neutrophils compared to macrophages (Fig. 4.1a) and when analysed by qRT-PCR OPN expression was around 75 times lower than in RAW 264.7 cells and about 25 times less than in peritoneal macrophages (not shown). The distribution of OPN
and its relationship to CD44 in neutrophils was also markedly different from that observed in activated macrophages and in fibroblasts (Zhu et al, 2004; Alstergren et al, 2004) (Fig. 4.1b). OPN expression (green) in neutrophils was observed in a punctate pattern throughout the cytoplasm while CD44 expression (red) localized to one end (uropod) of polarized neutrophils. Thus, as observed previously (Alstergren et al, 2004), there appears to be little specific association between the OPN and CD44 apparent in neutrophils, and the lack of OPN expression did not influence the expression of CD44. Moreover, staining for F-actin showed no difference in stress fibre formation or in the size or shape of polarized neutrophils (Fig. 4.1c).

**Analysis of neutrophil chemotaxis and chemokinesis**

To determine whether OPN functions in the migration of neutrophils, studies of chemokinesis and chemotaxis were performed in modified Boyden chambers (Transwell chambers®) and in Zigmond chambers. Addition of the chemoattractant fMLP ($10^{-6}$ mol/l) to the lower side or both sides of the Transwell chambers increased both chemotactic and chemokinetic migration of freshly isolated WT neutrophils, respectively (Fig. 4.2a). Whereas the basal migration (PBS control) was not significantly different from WT cells, both chemotaxis and chemokinesis were markedly reduced in the OPN$^{-/-}$ neutrophils. The impaired chemotaxis of the OPN$^{-/-}$ cells could be rescued dose-dependently by the addition of exogenous OPN to 0.25 μg/ml. However, at higher concentrations (1.0 μg/ml), the effect of OPN was reduced. Chemotaxis was also suppressed by the addition of OPN to the WT neutrophils, indicating that OPN was exerting a chemotactic effect that competed with the response of the cells to the fMLP.
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(a)

- **676 bps**
- **517 bps**
- **460 bps**
- **396 bps**

(b)

- **CD44**
- **OPN**
- **Merged**
- **Phase**

(c)

- **WT**
- **OPN⁻⁻⁻**
Figure 4.1. Expression of OPN in neutrophils. (a) OPN mRNA levels in neutrophils (PMNs) were measured by RT-PCR and compared with OPN expression by RAW 264.7 macrophages, using β-actin as a control. A single 408-bp amplicon was amplified with much lower amounts in the neutrophils (+ RT). Only a trace of product was observed in the absence of reverse transcriptase (– RT). Quantitative PCR revealed that OPN mRNA in the neutrophils was 75-fold less than in the RAW 264.7 cells. (b) Confocal immunofluorescence analysis of polarized neutrophils stained for cell-surface surface (non-permeabilized cells) CD44 (red) and cellular (permeabilized cells) OPN (green). No significant colocalization of the punctate OPN with the CD44, which is concentrated in the uropod, was evident. (c) Immunofluorescence staining of neutrophils with TRITC-phalloidin to examine F-actin distribution showed no differences between the OPN+/− null and WT cells regardless of their polarization state.
gradient. That OPN is chemotactic for neutrophils was demonstrated by a dose-dependent increase in migration for both the WT and OPN−/− neutrophils when OPN was added to the lower chamber of the Transwell system (Fig. 4.2b).

**In vitro neutrophil polarization and migration**

Migration analyses in Zigmond chambers allowed cell polarization and migration speed to be analysed, together with measurements of chemotaxis by individual cells (Koh et al, 2005). The proportion of WT and OPN−/− neutrophils that polarized in any direction (random) and in the direction of the chemoattractant was examined before and during the first 15 min of fMLP stimulation (Fig. 4.3a). While the proportion of WT and OPN−/− neutrophils that migrated randomly and directionally after 15 min was similar for both phenotypes there was a much lower proportion of directionally migrating cells compared to randomly migrating cells (Fig. 4.3a). The addition of macrophage OPN (1 μg/ml and 2.5 μg/ml) to OPN−/− cells increased both random and directed migration reproducibly but with low significance \((P < 0.1)\). When the cells were analysed for speed of polarization, there was little difference for random polarization, whereas directed polarization to fMLP was always less in the OPN−/− cells (Fig. 4.3b). Although these differences were not significant at the \(P < 0.05\) level at any time-point, they were a consistent finding in replicate experiments. In the presence of exogenous OPN the speed of polarization of OPN−/− cells was increased, but this was significant only for random polarization \((P < 0.05)\). In agreement with the Transwell assays, OPN−/− neutrophils had a slower migration speed, which was markedly increased in the presence of exogenous OPN \((P < 0.001)\) (Fig. 4.3c).
Figure 4.2. Migration of neutrophils in transwell chambers. Neutrophils were analysed for migration in response to $10^{-6}$ mol/l fMLP stimulation and OPN using BSA as a control. The number of neutrophils migrating through the 3.0-μm pores after 30 min in Transwell chambers was determined and the means ± SD were calculated for triplicate samples. (a) Migration toward fMLP, which was consistently decreased in OPN−/− neutrophils, could be partially rescued by low concentrations of exogenous macrophage OPN added to both sides (BS) of the membrane, but decreased migration in both WT cells and in OPN−/− cells at higher concentrations. (b) A dose-dependent increase in migration towards OPN added to the lower chamber (LC) was observed for both WT and OPN−/− cells, the OPN−/− neutrophils responding more strongly. The results from one of three replicate experiments are shown.
Recruitment of neutrophils \textit{in vivo}

Since OPN has been shown to be required for normal migration of macrophages, we first studied the recruitment of neutrophils into the peritoneal cavity using sodium periodate. The total number of cells recovered in the peritoneal lavage following periodate stimulation was more than two-fold lower in OPN-null mice and the percentage of neutrophils recovered was more than three-fold lower than in the WT controls (Fig. 4.4a), indicating that the absence of OPN has an impact on the recruitment of neutrophils. In contrast, the proportion of other immune cells, such as monocytes and lymphocytes, was not lowered. Notably, no differences in the total number of cells recovered, or in the fractions of different populations including neutrophils, were observed following longer stimulation (48 hr) with 1% Brewer's thioglycollate medium (not shown), which is used for macrophage peritoneal preparations (Zhu et al, 2004). The importance of exogenous OPN for the \textit{in vivo} recruitment of neutrophils was demonstrated by peritoneal injection of both native and recombinant OPN (Fig. 4.4b) inducing a peritoneal infiltrate comprising predominantly PMNs (> 65% and 90%, respectively) whereas lower numbers of PMN (< 3%) were present in the vehicle-injected controls.
Figure 4.3. Polarization and migration of neutrophils in a Zigmond chamber. (a) The proportion of neutrophils that polarized randomly and in the direction of fMLP was determined after 15 min. Fewer cells were seen to display directed migration for both WT and OPN−/− neutrophils. Addition of exogenous macrophage OPN to OPN−/− cells increased both random and directed migration reproducibly, but not significantly (P > 0.1). (b) Analysis of neutrophils over the 15-min time interval consistently showed a lower directed polarization for the OPN−/− cells, but the differences were not statistically significant. Exogenous OPN increased both random and directed polarization to similar levels observed in WT neutrophils but the increases were only significant (*P < 0.05) for the random polarization. (c) The average migration speed of OPN−/− neutrophils was lower than the WT cells (*P < 0.05). While fMLP stimulation increased the average speed of both WT and OPN−/− neutrophils, the effect was less in the OPN−/− neutrophils. Addition of exogenous OPN increased migration of OPN−/− cells approximately three-fold (**P < 0.001). Results are expressed as means ± SD.
**Phagocytosis assays**

To determine whether the functional activities of neutrophils were affected by the absence of OPN expression, superoxide and matrix metalloproteinase production were determined together with their phagocytic activity. The general phagocytic activity of the neutrophils was analysed by incubating neutrophils with fluorescent beads and determining the percentage of neutrophils with phagocytosed beads. Selective phagocytosis through both Fcγ and complement receptors was analysed by incubating neutrophils with sRBCs coated with IgG and IgM ligands, respectively. No impairment was evident in the ability of neutrophils derived from OPN-null mice to phagocytose uncoated beads, or ligand-coated beads (Fig. 4.5). Instead, there was an indication that specific phagocytosis was increased in the OPN–/– cells, suggesting that OPN expression in neutrophils is not required for either general or specific phagocytosis.

**Superoxide production**

Production of superoxide, which is an integral part of the bactericidal activity of neutrophils (Roos et al, 2003), was measured by NADPH oxidase activity, which generates H₂O₂. Neutrophils were stimulated with either LPS or fMLP and NADPH oxidase activity was measured as mean fluorescence intensity after 15 min. No significant differences in superoxide production were evident between the WT and OPN–/– neutrophils with and without stimulation by LPS or fMLP (Fig. 4.6a). The lack of differences was verified by normalizing the data to the unstimulated controls for each cell type (Fig. 4.6b).
Figure 4.4. Recruitment of neutrophils *in vivo*. The total number of immune cells in peritoneal exudates following intraperitoneal injections of 5 mM periodate or 20 μg/ml OPN was determined in Wright–Giemsa-stained slides using a haemocytometer. (a) Recruitment of immune cells by periodate was more than three-fold lower (open bars) in OPN−/− null mice with a disproportionately lower recruitment of neutrophils (black bars). (b) In response to administration of full-length native or recombinant OPN an infiltrate containing predominantly neutrophils was obtained, whereas PBS vehicle control did not recruit any significant numbers of neutrophils. Results are expressed as mean ± SD.
Figure 4.5. Phagocytosis assays. The ability of LPS-stimulated neutrophils to phagocytose polystyrene beads was assessed by incubating cells for 25 min at 37°C with fluorescent beads and the number of beads internalized by neutrophils was determined by flow cytometry. No differences between cells derived from OPN−/− null mice or WT controls in the phagocytosis of beads were observed. Specific phagocytosis mediated through Fc or complement receptors (C5) was analysed using red blood cells labelled with Texas Red–sulphonyl chloride dye. No significant differences were observed between cells derived from OPN−/− mice or WT controls in the phagocytosis of beads. Samples from three separate experiments were analysed by fluorescence microscopy and flow cytometry and the results are expressed as the percentage of cells containing one or more beads (mean ± SD).
Analysis of cytokines and matrix metalloproteinase 9 (MMP-9)

Cytokine production by neutrophils is an integral part of the innate immune response. The cytokines are stored in granules and released when neutrophils are activated. The cytokines released by PMA in WT and OPN\(^{+/−}\) were assessed using a cytokine blot (Panomics). Although the amounts of different cytokines varied, with higher levels of interferon-\(γ\) (IFN-\(γ\)), TNF-\(α\), interleukin-1\(α\) (IL-1\(α\)) and IL-4, there were no significant differences in the pattern of cytokines released by WT and OPN\(^{+/−}\) neutrophils (Fig. 4.7a). Neutrophils characteristically produce the gelatinase, MMP-9, which functions in inflammation-associated tissue destruction (Peake et al, 2006) that can be attenuated by the selective inhibition of MMP-9 (Kim et al, 2006). MMP-9 was analysed in cell extracts and conditioned PBS with and without de-granulation with PMA, as shown in a representative enzymogram (Fig. 4.7b). The majority of enzyme was present inside the cells in the latent, 92 000 molecular weight pro-MMP form, with only a trace of activated MMP-9. More than half of the pro-MMP was released by PMA treatment of the neutrophils. However, no significant differences were observed in the activation or release of pro-MMP-9 by PMA treatment of WT and OPN\(^{+/−}\) cells.
**Figure 4.6.** Neutrophil production of H$_2$O$_2$ was assessed from the mean fluorescence intensity (MFI) of rhodamine-123 and used as a measure of the oxidative burst. Samples were analysed by fluorescence microscopy and flow cytometry (mean ± SD). (a) No differences were observed between the WT and OPN$^{-/-}$ neutrophils stimulated with either fMLP or LPS. (b) The ratio of fluorescence between stimulated and unstimulated PMNs confirmed the lack of significant differences in oxidative burst ability.
**Figure 4.7.** Cytokine release and gelatin enzymography. Neutrophils were incubated with 0.1 µM PMA to stimulate neutrophil degranulation. (a) Cytokines released into PBS were analysed on a cytokine blot. The key to duplicate spots for cytokines and both positive (PC) and negative (NC) controls is shown in the right panel. No differences in the release of cytokines by WT and OPN−/− cells were evident in these assays. (b) Gelatinolytic enzymes in cell extracts and in conditioned PBS with and without stimulation with PMA were analysed by enzymography. Only pro-MMP-9 and activated MMP-9 were detected, together with an unidentified slower migrating protein with gelatinase activity. Most of the activity corresponded to the pro-MMP-9, which was effectively released into the PBS by the PMA treatment. No differences in MMP-9 expression between the WT and OPN−/− neutrophils were evident in replicate experiments.
DISCUSSION

Ablation of OPN expression has been shown to reduce the magnitude of the immune response in several models of inflammatory diseases including arthritis (Yumoto et al, 2002), multiple sclerosis (Chabas et al, 2001), kidney disease (Noiri et al, 1999) and lung fibrosis (O’Reagan et al, 2001), in which granuloma formation and T-cell responses predominate in the established inflammatory lesion. Although it is evident that OPN produced by macrophages and T lymphocytes has an important role in cell-mediated immunity, its function in neutrophils in the early stages of the innate response has not been reported. To determine the basis of the exacerbated inflammatory destruction observed in experimental colitis of OPN-null mice, which is associated with an increase of myeloperoxidase activity (Da Silva et al, 2006), we investigated the effects of OPN on neutrophil function. These studies have revealed defective recruitment of neutrophils in OPN-null mice, which may relate to impaired chemotaxis or the requirement for exogenous OPN. However, the functional activities of the OPN−/− neutrophils, including cytocidal activities, phagocytosis, generation of reactive oxygen species (ROS; oxidative burst), cytokine and MMP-9 production, were not impaired. Thus, while OPN may be important for neutrophil recruitment, in experimental colitis the rich blood supply to the gut is likely to circumvent this deficit and provide enough neutrophils in the inflamed tissues to compensate for the impaired activity of macrophages and lymphocytes (Da Silva et al, 2006). Moreover, the elevated destruction of the inflamed gut tissues can be explained by the persistence of the neutrophils (Haslett et al, 1994) because of the reduced clearance by the defective macrophages in the OPN-null mice (Sodek et al, 2006).
Neutrophils are highly motile cells that function in the immediate innate immune response, phagocytosing foreign materials and protecting the host by production of cytotoxic agents such as ROS and secretion of proteolytic enzymes. In regulated innate responses neutrophils are active at the injured site for up to 24 hr, after which time they undergo apoptosis and are cleared by macrophages. In certain inflammatory pathologies, such as oxidative stress, neutrophils can persist or exhibit hyperactivity, resulting in excessive damage to the injured tissue (Naito et al, 2003; Teder et al, 2002; Kruidenier et al, 2003; Jaeschke and Smith, 1997). Recent studies have shown that OPN\textsuperscript{−/−} macrophages exhibit cytoskeletal defects that affect their migration (Zhu et al, 2004) and exhibit impaired cytokine production, survival and phagocytic activity (authors unpublished). However, neutrophils express much lower amounts of OPN in comparison to macrophages (Fig. 4.1) and secretion of OPN by neutrophils could not be demonstrated in our studies. Moreover, unlike macrophages, neutrophils do not demonstrate an intracellular association of OPN with the CD44 receptor and the morphological changes reflecting F-actin rearrangements seen in OPN\textsuperscript{−/−} macrophages (Zhu et al, 2004) were not evident in OPN\textsuperscript{−/−} neutrophils. Thus, if neutrophils express an intracellular form of OPN (Sodek et al, 2002) that is thought to regulate the formation of cell processes and cell motility in fibroblasts (Zohar et al, 2000) and macrophages (Zhu et al, 2004; Suzuki et al, 2002), and has been observed to regulate IFN-α production in plasmacytoid dendritic cells (Shinohara et al, 2006), the association with CD44 and effects on the cell cytoskeleton are not evident. As discussed previously (Alstergren et al, 2004), the difference in OPN association with CD44 may reflect differences in the basic mechanisms involved in the migration of neutrophils, which express lower levels of
integrins (Friedl et al, 1998). Thus, in neutrophils, which migrate more rapidly than macrophages, the expression and localization of CD44 is largely independent of OPN (Sodek et al, 2006), (Fig. 4.1), suggesting that OPN may function differently in these cells.

That OPN−/− neutrophils exhibit impaired acute/short-term peritoneal recruitment probably relates to the observed defects in chemotactic and chemokinetic migration, which appear to be associated with the absence of exogenous OPN (Figs 4.2–4). Although the recovery of total inflammatory cells in the peritoneal lavage was significantly lower in OPN-null mice, there was a disproportionate decrease in neutrophils compared to monocytes and lymphocytes (Fig. 4.4a) in response to periodate stimulation. Since marrow preparations of neutrophils for the in vitro analyses did not show any difference in cell counts in general, or neutrophils in particular, when the WT and OPN−/− cells were compared (data not shown), the lowered fraction of neutrophils following acute stimulation does not appear to represent a defect in the production of neutrophils in the marrow. Notably, no decrease in the proportion of neutrophils was evident in the peritoneum 48–72 hr after administration of dithioglycollate, indicating an impairment in the initial recruitment of neutrophils, but not in the subsequent accumulation of cells. Consistent with the robust and selective recruitment of neutrophils into the peritoneum (Fig. 4.4b) and the demonstration that neutrophils are the main cell to be recruited by extracellular OPN in alcohol-induced hepatitis (Banerjee et al, 2006), our in vitro studies showed that exogenous OPN is chemotactic for neutrophils (Fig. 4.2b) and, while providing a partial rescue of polarization, markedly increased migration.
Thus, the impaired recruitment of the OPN−/− neutrophils in the OPN-null mice can be attributed to the lack of both chemotactic and migration effects on the neutrophils.

Although it is difficult to separate the effects of exogenous OPN from the OPN expressed by the neutrophils to identify the role of the neutrophil OPN, it is clear that the exogenous OPN has a more prominent effect on neutrophil recruitment. Moreover, the exogenous OPN, which is released by resident and immune cells at sites of injury, appears to exert its functions through the CD44 receptor. This is indicated by the ability of recombinant OPN, lacking post-translational modifications, to show the same efficacy as native OPN for peritoneal recruitment (Fig. 4.4b), and our previous demonstration that CD44 receptor expression is important for the directed migration and polarization of neutrophils (Alstergren et al, 2004). Notably, post-translational modifications have been shown to be important for OPN signalling through integrins but not for CD44-mediated activities (Ashkar et al, 2001).

In contrast to the defects in motility, OPN−/− neutrophils did not show any impairment in their phagocytic, cytotoxic, matrix degradative activities or cytokine expression. Thus, unspecific phagocytosis of fluorescent beads, and receptor-associated phagocytosis through Fc or complement receptors, was equally efficient in the OPN−/− neutrophils, with some higher values for the OPN−/− neutrophils (Fig. 4.6). As phagocytosis is a cytoskeleton-dependent process, in which re-organization of their cytoskeleton in response to stimuli is required to extend membranal protuberances supported by F-actin, the absence of cytoskeletal defects in the OPN−/− cells (Fig. 4.1b) is in agreement with the lack of defects in phagocytosis. Similarly, ROS production and
MMP-9 production are not impaired in the OPN−/− neutrophils (Figs 4.6 and 4.7). The ability to generate ROS is one of the important killing mechanisms of neutrophils and impaired ROS generation results in pathologies such as chronic granulomatous disease (Roos et al, 2003). MMP-9 is a prominent matrix-degrading enzyme typically expressed at high levels in activated neutrophils. The gelatinase activity expressed by neutrophils has a marked impact in destructive inflammatory diseases and is used as a marker for disease activity (Peake et al, 2006), while its selective inhibition can protect against inflammatory injury by neutrophils (Roos et al, 2003). The sustained production of ROS and MMP-9 by neutrophils is likely to be responsible for the massive destruction of the intestinal crypts when colitis is induced in OPN-null mice (Da Silva et al, 2006).

In summary, these studies have demonstrated the importance of OPN expression in general and exogenous OPN in particular for the migration and recruitment of neutrophils. However, no significant affects of OPN were observed on the phagocytic, cytotoxic or matrix degradative activities of neutrophils. Thus, while the initial recruitment of neutrophils may be impaired, their destructive effects are not limited by OPN expression and are likely to be highly dependent on their removal from inflammatory sites.
CHAPTER 5: Osteopontin Attenuation of Dextran Sulfate Sodium-induced Colitis in Mice


Osteopontin Attenuation of Dextran Sulfate Sodium-induced Colitis in Mice. Submitted Laboratory Investigation.
ABSTRACT

Osteopontin (OPN) is a matricellular cytokine present in most tissues and body fluids; it is known to modulate immune responses. In previous studies using the dextran sulfate sodium (DSS) acute colitis model, we found exacerbated tissue destruction and reduced repair in OPN-null (-/-) mice compared with wild-type (WT) controls. Since OPN is normally present in milk, we hypothesized that administration of OPN may protect the intestines from the adverse effects of experimental colitis. Twenty µg/ml or 2 µg/ml bovine milk OPN, dissolved in drinking water, was given to mice 24 h prior to, and during, administration of DSS. Clinical parameters of colitis and neutrophil functions were analyzed as previously reported. Orally administered OPN was absorbed and detected in the colon mucosa by immunohistochemistry. The 20 µg/ml OPN- and DSS-treated WT mice exhibited 37 % less weight loss and reduced colon shortening and spleen enlargements than control mice (p<0.05). OPN administration also reduced the disease activity index, improved red blood cell counts, and reduced gut neutrophil activity compared with the DSS-treated WT mice that were not administered OPN (p<0.05). Immunohistochemical detection of F4/80- labelled cells (macrophages) was also less frequent. The level of transforming growth factor beta 1 (TGF- β1) was increased and the levels of pro-inflammatory mediators decreased in colon tissue samples of OPN-treated mice analyzed by ELISA. The reversal of experimental colitis parameters by exogenous OPN was not as robust in the OPN-/- mice. Administration of prokaryotic-expressed recombinant OPN and bovine serum albumin were ineffective. This study demonstrates that administration of a physiologic concentration of milk OPN in drinking water ameliorates the destructive host response in DSS-induced acute colitis.
INTRODUCTION

The pathogenesis of inflammatory bowel disease, which includes ulcerative colitis and Crohn’s disease, is believed to involve increased host susceptibility combined with de-regulated immune responses to microbial and dietary antigens (Orlando et al, 2002; Podolsky et al, 2002a; Loftus et al, 2004). During colitis, neutrophils migrate into the lesion, and they are found within intestinal crypts and at the base of ulcerations, forming crypt abscesses (Stevceva, 2001). Furthermore, several studies have shown that the total number of macrophages increases, including some subpopulations of macrophages that are not normally present in the lamina propria of the intestine (Hume et al, 1987; Oshitani et al, 1996). Macrophages together with neutrophils may contribute to intestinal damage by releasing radicals derived from oxygen metabolism (Britigan et al, 1988) and by secreting proinflammatory cytokines and other inflammatory mediators. For our studies, we used the DSS-induced colitis model, which was first described by Okayasu et al (1990), and which has been extensively used in IBD studies. This murine model exhibits a deregulated inflammatory response coincident with many pathological changes that are similar to those seen in human ulcerative colitis (Okayasu et al, 1990; Cooper et al, 1993). While the lesions are rather uniform and reproducible, the time course and severity of the induced colitis mimics that seen in humans (Murthy et al, 1993). Consequently, DSS-induced murine colitis has been recognized as a valuable tool in the identification of potential therapeutic agents, and it is considered to be a sensitive screening system (Elson & Weaver, 2007).

Osteopontin (OPN) is a matricellular cytokine present in most tissues and body fluids. It has been suggested that OPN has an important modulatory role in innate
immunity, which is reflected by its mucosal protective functions in some infectious
diseases (reviewed in Wang & Denhardt, 2008). OPN is secreted by dendritic cells,
activated macrophages, T-lymphocytes, and subsequently by proliferating fibroblasts
during reparative matrix formation (Ashkar et al., 2000; O'Regan & Berman, 2000;
Kawamura et al., 2005). We have shown that OPN is upregulated in epithelial cells of the
intestinal mucosa during experimental colitis (Da Silva et al., 2006). We have also shown
that OPN is chemoattractive for neutrophils but not involved in phagocytosis or
generation of reactive oxygen species (Koh et al., 2006). Yet, OPN regulates macrophage
functions such as migration, activation, and phagocytosis (Zhu et al., 2004; Roloo &
Denhardt, 1996; McKee & Nanci, 1996). OPN-mediated initiation of healing appears to
be associated with upregulation of TGF-β1, which induces restitution of the intestinal
epithelial barrier, decreases apoptosis of fibroblasts, and results in increased matrix
deposition (Dignass and Podolsky, 1993; Desmoulière, 1995; Jelaska and Korn, 2000;
Zohar et al., 2004).

Our previous study using the experimental acute colitis model found a greater
susceptibility of OPN-/- mice than wild-type (WT) mice to DSS-induced colitis (Da Silva
et al., 2006). Clinical parameters of colitis, such as weight loss, disease activity scores,
colon shortening, and spleen enlargement were significantly greater in OPN-null mice
and accompanied by an exacerbation of intestinal tissue damage. Tissue destruction was
associated with the persistence of neutrophils and reduced survival of enterocytes.

Though OPN has been associated with a detrimental type 1 helper T cell immune
response in other inflammatory diseases (Miyazaki et al., 1995; Noiri et al., 1999; Chabas
et al., 2001; O'Regan et al., 2001; Yumoto et al., 2002), it had important protective
functions in our studies of acute colitis. OPN may modulate local immune responses by impacting innate immune cell functions, expression of inflammatory cytokines and thereby attenuating inflammatory tissue damage. This raised the question whether dietary exposure to exogenous OPN may be sufficient to restore an experimental colitis immune response that is less damaging. Therefore, we hypothesized that exogenous delivery of milk OPN in drinking water of mice may protect the colon from the adverse effects of DSS-induced inflammation. Our data show that bovine milk OPN at 20 µg/ml concentration, administered for 8 days in drinking water, ameliorated DSS-induced colitis in mice. It reduced the levels of innate immune cells and pro-inflammatory mediators in the diseased colon and diminished the classic hallmarks of the disease.

MATERIALS AND METHODS

Source and biotinylation of osteopontin

Samples of bovine milk OPN, containing a mixture of intact OPN and OPN-derived peptides, were prepared as described (Sørensen & Petersen, 1993). For preparation of recombinant OPN (r-OPN), a pET28a expression vector containing rat OPN cDNA was used to transform Escherichia coli BL21 cells. The recombinant protein was purified by nickel affinity chromatography and fast protein liquid chromatography as previously described for bone sialoprotein (Tye et al., 2003). Biotinylated bovine milk OPN (b-OPN) was prepared as follows: 0.5 mg/ml OPN in a 50 mM sodium bicarbonate buffer (pH 8.5) was mixed with biotin-N-hydroxysuccinimide ester dissolved in dimethyl sulfoxide for 2 hr at 4 ºC, desalted on a 10 ml desalting column (PD-10 desalting columns, Amersham Biosciences Piscataway, NJ) equilibrated in 50 mM ammonium
bicarbonate buffer (pH 8.5). A dot blot assay with streptavidin HRP was used to confirm the fractions containing OPN. The OPN was dried on a speed vacuum and reconstituted in phosphate-buffered saline (PBS), pH 7.2, at a concentration of 1 mg/ml.

**DSS-induced experimental acute colitis in mice and delivery of exogenous OPN**

Approved animal experiments were conducted according to guidelines established by the Animal Care Committee of the University of Toronto. The generation and initial characterization of OPN-null mice was described previously (Rittling et al., 1998). The original 129sv F2 mice were subsequently back-crossed (10×) into a C57BL/6J background. Polymerase chain reaction (PCR) was used at regular intervals for genotyping of OPN-/- mice to confirm that the colony had not been contaminated. For these studies, we used adult OPN-/- males, 8 to 10-week-old, and their matched C57BL/6J wild-type (WT) controls. The mice were housed in a specific pathogen-free facility with controlled temperature and light/dark cycles, and fed standard mouse chow pellets. DSS (DSS - mol wt. 36,000-50,000; MP Biomedical, Inc., Aurora, OH) 5% (wt/vol), dissolved in sterile distilled water, was given for 7 days to induce experimental colitis (Cooper et al, 1993). Control animals were given water only, and water consumption was monitored for both animal groups. Bovine milk OPN was dissolved in the drinking water at 20 µg/ml, representing the normal physiological concentration in bovine milk, or at 2 µg/ml. For short-term experiments to determine whether OPN would be detected in the colon tissues and in the blood following exogenous administration of OPN, a higher dose of 200 µg/ml, which approximates the physiological concentration of human milk, was also used. The OPN was continuously given to mice starting 24 hours
prior to administration of the DSS to induce colitis. The mice were sacrificed after 7 days of DSS, which was the 8th day of OPN administration. In one set of experiments, bovine serum albumin (BSA) was used in place of OPN as an exogenous protein control. Similarly, the therapeutic impact of administering prokaryotic-expressed recombinant OPN, lacking typical post-translational modifications, was compared to that of the exogenous milk OPN.

**Analysis of clinical parameters**

Clinical parameters were analyzed as previously reported (Da Silva et al, 2006). Among other key variables (body weight, spleen enlargement, colon length, and water consumption), the parameters included variables originally described by Hartmann et al (2000) as a “disease activity index (DAI)”, based on anal bleeding and the nature of the feces. Individual animal scores between 0-3 were used, with ‘0’ referring to normal stool and no fecal blood, and ‘3’ for liquid stool and/or presence of blood on the cage wall. The mean and standard error of the mean were determined for each group.

**Detection of biotinylated OPN and murine OPN in tissue sections by immunohistochemistry**

The b-OPN and DSS were dissolved in the drinking water, and the mice were sacrificed after 7 days of DSS treatment, as described above. The b-OPN was detected in sections of colon tissues by immunohistochemistry as previously described (Da Silva et al, 2006). Briefly, frozen sections were fixed in 50 % acetone, placed in ethanol for 10 min and stored at -20 ºC for two days. The sections were incubated with 3 % H$_2$O$_2$ for 10 min to
eliminate endogenous peroxidase activity, and blocked with 1 % BSA for 10 min. Sections were incubated with avidin and biotinylated horseradish peroxidase (HRP) (Vectastain Universal Elite ABC kit) for 30 min, and then the HRP enzyme activity was visualized by adding the chromogenic substrate diaminobenzidine tetrachloride (DAB; Vector Laboratories, Burlingame, CA) for 10 min, which results in brown staining. The expression of OPN in mouse colons was also examined by immunohistochemical analysis. The sections were incubated overnight with a rabbit anti-porcine OPN antibody, (Zhang et al, 1990) affinity-purified using recombinant rat OPN, and sections were incubated with goat anti-rabbit IgG for 30 min.

**Plasma b-OPN quantification**

Biotinylated milk OPN, 20 µg/ml in drinking water, was ingested ad libitum by 12 mice for 8 days. Eight additional mice were selected and treated with 1 ml of 20 µg/ml or 200 µg/ml by oral gavage. After 4 h, blood was collected from all the mice, and the resulting plasma stored at -80 ºC. A modified sandwich ELISA was used to quantify the amount of b-OPN in the samples. Briefly, 100 µl of 4 µg/ml polyclonal rabbit antibody raised against bovine milk OPN was added to the wells of an enhanced protein-binding ELISA plate (Falcon cat. no. 353279). As a negative control, some wells were left coating-free. After incubation overnight at 4 ºC, the plate was brought to room temperature, the capture antibody solution was removed, and the wells blocked by the addition of 200 µl of 0.05 % Tween-20 and 1 % BSA in PBS for 1 hr. After washing 3 times with
PBS/Tween, 100 µl of a plasma sample was added and incubated for 4 h at room temperature. The streptavidin-HRP conjugate was used at a dilution of 1:1000 in PBS. The substrate was added and absorbance at 490 nm determined (Titertek Multiskan reader; Titertek Instruments, Huntsville, AL).

**Peripheral blood cell analyses**

Approximately 0.5-1 ml of blood was collected by intracardiac puncture, placed in a labeled vial containing 1,000 µ/ml heparin, and sent on ice for hematological analysis to VITA-TECH Laboratories (Toronto, Ontario). Blood samples were analyzed for total blood cells and differential white blood cell (WBC) counts, red blood cell (RBC) counts, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular volume and mean corpuscular volume concentration.

**Myeloperoxidase (MPO) activity**

MPO activity was analyzed in gut tissue samples as a surrogate variable to evaluate neutrophil accumulation and activity, using a commercial kit for the o-dianisidine assay (Cytostore, Calgary, AB). Briefly, weighed gut strips that had been washed in PBS and stored at -80 °C were thawed, homogenized in a 0.5 % hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0), and clarified by centrifugation for 2 min at 9,000 g at 4 °C. An aliquot of the supernatant was allowed to react with the chromogen (o-dianisidine dihydrochloride) in the presence of potassium phosphate buffer and 1 % H₂O₂, as supplied by the manufacturer. The absorbance at 450 nm was measured immediately by spectrophotometry and again at 60 sec after the addition of the
chromogen to determine the reaction rate. Murine bone marrow neutrophils, isolated on a Percoll gradient, served as a positive control. MPO was expressed as the quantity of enzyme in 1 mg tissue that degraded 1 µmol/min of peroxide at 37 °C.

**Macrophage detection using F4/80 antibody staining**

F4/80 antigen expression by macrophages was detected by immunohistochemistry using a rat monoclonal F4/80 antibody (ab6640, Abcam, Cambridge, MA) to label sections of colon tissues. Briefly, frozen sections were fixed in 50 % acetone, placed in ethanol for 10 min and stored at -20 °C. The sections were incubated with 3 % H₂O₂ for 10 min to eliminate endogenous peroxidase activity. After blocking with Rodent Block M (Cat# RBM961L, Biocare Medical LLC, Concord, CA) for 30 min, the sections were incubated for 60 min at room temperature with 1:50 diluted rat anti-mouse F4/80 antibody. Sections were incubated with anti-rat IgG (Rat on Mouse HRP-Polymer Kit, Cat# RT517L, Biocare Medical LLC, Concord, CA) for 30 min, and then the chromogenic substrate (DAB) for 10 min. Mouse spleen was used as the positive control; the primary antibody was omitted for the negative control.

**Quantification of transforming growth factor beta 1 (TGF-β1) and cytokine assay**

The colon tissue samples were prepared as described above for the MPO assay, and aliquots were analyzed for TGF-β1 content. The TGF-β1 concentration was determined by a quantitative sandwich enzyme immunoassay technique using a commercial kit (Quantikine, Minneapolis, MN). In addition, the homogenized gut tissue samples were analyzed with an antibody-based protein array system to quantify secreted pro-
inflammatory cytokines, such as granulocyte colony-stimulating factor (G-CSF), interferon gamma (IFN-γ), macrophage colony-stimulating factor (M-CSF), tumor necrosis factor alpha (TNF-α), monokine induced by gamma-interferon (MIG), regulated on activation normal T cell expressed and secreted (RANTES), using Tran-Signal TM mouse cytokine antibody arrays (Panomics, Fremont, CA). Changes in cytokine secretion were evaluated for each sample in relation to the mean density of the negative controls (0 %) and the mean density of the positive controls (100 %), supplied by the manufacturer, on each cytokine array membrane. The values expressed as the percentage change (mean ± SD).

**Statistical analyses**

Results are expressed as mean ± standard error of the mean (mean ± SEM). Experiments in vivo were performed at least five times with three or more mice in each group, and analysis of tissues was performed at least three times. The data were compared using one-way ANOVA analysis with the Bonferroni method as post hoc test. Statistical significance was defined as $P < 0.05$. Calculations were performed using the statistical analysis software SPSS (SPSS, Inc., Chicago, IL).

**Results**

**Absorption of bovine milk OPN**

To determine whether bovine milk OPN was absorbed during the course of OPN treatment, 20µg/ml biotinylated-OPN was administered orally, and its concentration in
plasma determined using the ELISA method. b-OPN ingested ad libitum was not detected in the plasma of these mice. However, mice that also received either 20 µg/ml or 200 µg/ml OPN by gavage had approximately 145 ng/ml and ~1050 ng/ml of b-OPN in plasma, respectively (Fig. 5.1). No reactivity was detected in the plasma of control mice administered OPN that was not biotinylated, either orally or by gavage.

**Milk OPN detection in inflamed colons**

To investigate whether OPN was taken up by the diseased colon tissues, b-OPN was administered to DSS-treated mice. Immunostaining of controls, sham, and DSS-treated WT colon tissue (nonbiotinylated OPN) showed a slight staining in the epithelial cells of the mucosa that may have been due to endogenous enterocyte biotin (Fig. 5.2A, 2B, S1D, S2A) since immunostaining for OPN itself yield a much stronger labeling (Fig. 5.S2B). No staining was observed in the absence of the streptavidin HRP complex (Fig. 5.2C, S1C). Administration of b-OPN resulted in increased staining within the colon; it was especially intense in the epithelium of the mucosa (Fig. 5.2D) and greater than the staining following exogenous administration of control protein b-BSA (Fig. S1E). In order to better establish the architecture of tissue sections analyzed in figure 2, hematoxylin and eosin staining from comparable tissues is also provided (Fig. 5.S3A, B, C, D). Together, the results of these experiments showed that OPN was retained by the mucosa of the colon following exogenous administration of bovine milk OPN.
Figure 5.1. Detection of biotinylated (b-) OPN in peripheral blood. b-OPN was not detected in the plasma of mice administered 20 µg/ml OPN continuously. However, mice that received either 20 or 200 µg/ml OPN by gavage had approximately 145 ng/ml and ~1050 ng/ml of OPN in plasma, respectively (**, P < 0.001, n=5), indicating that the OPN can be absorbed through the gut wall.
Figure 5.2. Detection of biotinylated OPN by immunohistochemistry. **A, B:** Immunostaining of sham and DSS-treated WT colon tissue that was not exposed to bovine biotinylated-OPN yielded slight immunostaining in the epithelial cells of the mucosa. **C:** No staining in WT DSS-treated colon in the absence of streptavidin-HRP complex. **D:** Immunostaining of colon sections of mice that were fed b-OPN in drinking water yielded abundant labeling, especially in the epithelial layer of the mucosa.
Milk OPN has beneficial effects on clinical outcomes in experimental colitis

Treatment of WT and OPN-null mice with DSS produced clinical and histological signs of colitis as previously reported (Da Silva et al, 2006); yet the DAI scores for WT and OPN mice were not statistically different in the present data set (Fig. S5). DSS-treated WT mice lost weight from day 4 onward; in contrast, the DSS-treated mice that were administered 20µg/ml milk OPN lost significantly less weight (Fig. 5.3A; P<0.05, n=9). The DSS-treated OPN-/- mice also lost weight; yet, administering them OPN diminished their weight loss only on day 6, (P<0.05, n=9). Water consumption measurements showed that DSS-treated mice that received the 20µg/ml dose of OPN consumed more water (Fig. 5.3B; P<0.05, n=7). This finding suggests that they were less sick, even though their increased water consumption would coincidently also increase their exposure to DSS. The DAI scores based on stool consistency and anal bleeding was ~ 40 % lower on the sixth and seventh day for the 20 µg/ml OPN group (Fig. 3C; P<0.001, n=9). Spleen enlargement, a marker of systemic inflammation, was calculated in proportion to the body weight. In the DSS-treated WT animals, 20µg/ml OPN induced a smaller increase in the spleen weight (Fig 5.3D; P<0.05, n=9). Similarly, colon length, a morphometric measurement of the degree of colitis, was not as short in the DSS-treated mice that had been given 20 µg/ml OPN as that in the other DSS-treated groups, for both WT and OPN-/- mice (data not shown). For some clinical parameters measured, the 2 µg/ml dose of OPN tended to induce partially protective effects, though not to the same degree as 20 µg/ml OPN (Fig 5.3A, 3C). For most of the clinical indices analyzed, the administration of exogenous OPN led to the reversal of the measured parameters in DSS-
fed WT mice, but to either no effect, or much less of an effect, in the DSS-fed OPN-/- mice.

Heparinized peripheral blood collected by intra-cardiac puncture on day 7 contained lower levels of RBC, hemoglobin and hematocrit (not shown) in mice treated with DSS, which is indicative of hemorrhage. Blood from the mice administered 20 µg/ml OPN had greater levels of RBC and hemoglobin (Fig. 5.4A, 4B; ~25% increase; $P<0.05$, n=9) as well as a hematocrit increased by ~25% (not shown), indicating decreased loss of fecal occult blood. However, the DSS-treated OPN-/- mice did not exhibit any significant difference in these parameters upon administration of OPN (Fig. 5.4A, 4B). These data suggest that beneficial effects of OPN in ameliorating the progression of colitis are not as readily achievable in OPN-/- mice.

Differentiation analysis of WBC counts (Fig. 5.4C, 4D, 4E) showed a statistically significant decrease in the level of neutrophils in DSS-treated mice that were administered 20 µg/ml OPN. The neutrophil count was markedly decreased, by ~43% (Fig. 5.4E; $P<0.05$, n=9). In contrast, in the OPN-/- mice, administration of 20 µg/ml OPN actually increased WBC counts (Fig 5.4C). Differential analysis showed that both lymphocyte and neutrophil counts were elevated compared with DSS-treated controls (Fig. 5.4D, 4E).

**OPN administration reduces the destructive potential of neutrophils and leads to lower macrophage counts during experimental colitis**
Myeloperoxidase (MPO) activity, a potentially tissue-destructive indicator of neutrophil infiltration, was increased markedly in samples of colon tissue from DSS-treated mice, with a greater increase in the colons of OPN-/- mice (Fig. 5A). Notably, MPO was reduced in the colons of the WT mice that had been administered 20 µg/ml OPN (Fig. 5.5A, $P < 0.05$, n=7).

Similarly, staining of colon sections with F4/80, a marker for macrophages, found that administration of 20 µg/ml milk OPN apparently led to decreased macrophage recruitment. In the sham-control WT colon, F4/80+ staining was observed mostly in the submucosa (Fig 5.5B). As noted in the control (Fig. 5.5C), no staining was observed in the absence of primary antibody. As expected, due to crypt destruction and high levels of proinflammatory cytokines (Da Silva et al, 2006), immunostaining for F4/80 in DSS-treated WT mice showed a large increase in the number of macrophages throughout the mucosa and submucosa (Fig. 5.5D). Administration of 20 µg/ml OPN to DSS-treated mice decreased macrophage infiltration (Fig. 5.5E). In order to better establish the architecture of tissue sections analyzed in figure 5, hematoxylin and eosin staining corresponding to these sections is provided in the supplemental figures (Fig. 5.S4A, B, C, D).
Figure 5.3. Clinical progression of DSS-induced colitis in WT (left column) and OPN-/- mice (right column) after 8 days administration of milk OPN. A: DSS-treated WT mice administered 20 µg/ml OPN lost significantly less weight than the DSS-treated control mice on days 5, 6 and 7 (*, \( P<0.05, n=9 \)). The weight loss for the OPN null mice given 20 µg/ml OPN was significantly less than controls only on day 6 (*, \( P<0.05, n=9 \)). B: Water consumption measurements showed that OPN-administered mice consumed more water (*, \( P<0.05, n=7 \)). C: DSS-treated WT mice administered 20 µg/ml milk OPN exhibited DAI scores significantly lower on day 6 and 7 (**, \( P<0.001, n=9 \)). No statistically significant differences were found in the OPN-/- mice (n=9). D: In the DSS-treated WT animals, 20 µg/ml of OPN led to a smaller increase in spleen size (*, \( P<0.05, n=9 \)), which was not seen in the OPN-/- mice (n=9).
**Figure 5.4.** RBC and WBC analysis in the peripheral blood. **A, B:** DSS-treated WT mice exhibited low levels of RBC and hemoglobin. The administration of 20 µg/ml led to greater levels of RBC and hemoglobin (*, $P<0.05$, n=9). DSS-treated OPN-/- mice did not exhibit any significant difference when OPN was administered. **C, D, E:** In the WT mice, OPN intake appeared to reduce the levels of WBC counts from the high levels of WBC seen in the control DSS-induced colitis blood samples. Differential analysis showed that neutrophils were markedly decreased in the OPN 20 µg/ml group (*, $P<0.05$, n=9). In the OPN-/- mice, the 20 µg/ml dose of OPN intake led to higher WBC counts. Differential analysis showed that this increase occurred mainly due to lymphocytes (*, $P<0.05$, n=9).
Figure 5.5. Colon tissue MPO activity and F4/80 immunstaining of colon sections. A: Myeloperoxidase activity was reduced in the colon of both WT and OPN-/- mice exposed to the higher concentration of exogenous OPN; yet, significant only in the WT group (*, P<0.05, n=7). B: Control staining of DSS-treated WT colon in the absence of primary antibody. C: Staining of sham WT colon showing F4/80 positive cells mainly in the submucosa. D: Immunostaining of DSS-treated WT mice indicating increased infiltration of macrophages in both the mucosa and submucosa. E: Staining showing decreased macrophage infiltration in OPN-administered DSS-treated mice.
**Decreased inflammation is accompanied by increased levels of TGF-β during experimental colitis**

TGF-β1 is both a pro-fibrotic growth factor and an immunosuppressive mediator that acts through a variety of mechanisms, including downregulation of proinflammatory cytokines. Therefore, TGF-β1 expression in colon tissue lysates was compared among the OPN treatment and control groups. Colon tissue from DSS-treated WT mice, but not from OPN-/- mice, exhibited elevated levels of TGF-β1 (Fig. 5.6; \(P < 0.001\)). Yet, when mice were administered 20 µg/ml milk OPN, the DSS-treated mouse colons from both WT and OPN-/- mice had even greater levels of TGF-β1 than that of the DSS-treated mice that did not receive exogenous OPN (Fig. 5.6; WT, \(P < 0.001\); OPN-/-, \(P < 0.05\), \(n=5\)).

**Decreased inflammation upon OPN administration is coincident with diminution of pro-inflammatory mediators**

Extracts of gut tissues were analyzed for the presence of pro-inflammatory cytokines using cytokine antibody arrays. Values were calculated as a percentage of the positive controls (100 %) in each array. DSS-treated WT colons exhibited significantly greater amounts (two- to tenfold) of G-CSF, IFN-γ, M-CSF, TNF-α, MIG, RANTES than colons of the sham group (Fig. 5.7). Administration of 20 µg/ml OPN reduced the levels of these inflammatory mediators to that of the sham group, which received no DSS.
During DSS-induced colitis, WT colons exhibited higher levels of TGF-β1 (**, $P<0.001$), which was not seen in the OPN-/- colons. Both WT and OPN-/- mice that were administered milk OPN exhibited even higher levels of TGF-β1 in colon tissue (WT, **, $P<0.001$; OPN-/-, *, $P<0.05$, n=5).

**Figure 5.6.** Increased TGF-β1 levels in colon tissues upon administration of milk OPN.
Figure 5.7. Proinflammatory cytokines in the colon tissue. Values on the y-axis were calculated as a percentage of the positive control (100%) in each array. In colon tissue extracts of WT DSS-treated mice, administration of 20 µg/ml OPN reduced the amount of G-CSF, IFN-γ, M-CSF, TNF-α, MIG, RANTES to control levels.
Bovine serum albumin (BSA) and recombinant OPN fail to reproduce the beneficial effects of milk OPN

In order to exclude that the beneficial effects of OPN in experimental colitis were due to a general effect of administering exogenous protein in drinking water, a comparison of BSA and OPN administration was assessed in DSS-treated mice. As before, 20 µg/ml OPN decreased the clinical signs of colitis (DAI scores, neutrophils) (Fig. 5.S1A,B; \( P < 0.05, n=5 \)). In contrast, 20 µg/ml BSA had no effect in modulating the DSS-induced colitis (Fig. 5.S1A,B).

Since it is known that a variety of cellular responses to OPN are due to its post-translational modifications (reviewed in Kazanecki et al., 2007), disease progression of experimental colitis was analyzed in DSS-treated mice administered either milk OPN or recombinant OPN (r-OPN), which contains no post-translational modifications. After four days of DSS treatment, the mice administered milk OPN exhibited lower scores for the DAI than the mice administered r-OPN (Fig 5.8A; \( P < 0.05, n=5 \)). Peripheral blood analysis showed decreased levels of RBC and higher levels of WBC in the r-OPN group, indicating blood loss and acute inflammation (Fig 5.8B). Moreover, r-OPN failed to reduce neutrophil and lymphocyte counts as observed for milk OPN (Fig 5.8C). These results suggest that the post-translational modifications of milk OPN, such as phosphorylation (reviewed in Kazanecki et al., 2007), are crucial for achieving the beneficial effects of exogenous OPN in DSS-induced colitis.
**Figure 5.8.** Lack of protection upon administration of recombinant OPN (r-OPN). **A:** DSS-treated mice administered 20 µg/ml milk OPN exhibited less severe clinical signs of colitis than the mice administered r-OPN and DSS or DSS alone (*, $P<0.05$, n=5). **B:** Peripheral blood analysis found decreased levels of RBC and high levels of WBC in the r-OPN group, indicating blood loss and acute inflammation. **C:** r-OPN failed to reduce neutrophil counts compared with milk OPN (*, $P<0.05$, n=5) after 8 days of treatment. Neither OPN preparation affected the lymphocyte count significantly.
DISCUSSION

Bovine milk OPN attenuates acute DSS-induced colitis

In a previous study, we found a greater susceptibility of OPN-null than WT mice to DSS-induced colitis (Da Silva et al, 2006). This finding led us to ask whether administration of exogenous OPN may have a protective effect during DSS-induced colitis. Our major findings in this study support this contention. We found that OPN (20 µg/ml) introduced for 8 days dissolved in the drinking water was detected in the colon mucosa (Fig. 5.2). It ameliorated DSS-induced colitis in mice by diminishing signs of disease and by decreasing fecal occult blood loss, peripheral blood neutrophils, colon mucosa neutrophils, macrophages and pro-inflammatory cytokines. Yet, others have demonstrated that abrogation of the interaction between OPN and its receptors by antibodies specific for the SLAYGLR domain of OPN resulted in the amelioration of inflammatory diseases such as rheumatoid arthritis, hepatitis, and inflammatory ocular disease (Yamamoto et al, 2003; Diao et al, 2004; Kitamura et al, 2007, Saito et al, 2007). Our findings suggest that the pathogenesis of acute colitis induced by DSS is different from that of these other inflammatory diseases, since it is a mucosal disease in which an intact mucosal epithelial barrier is required to combat intermittent bacterial influx. Therefore, in contrast to DSS-induced chronic colitis, in which OPN may induce polarization of the type 1 helper T cell immune response to cause increased inflammation (Heilmann et al, 2008), OPN may actually attenuate inflammation in DSS-induced acute colitis by contributing to the resolution of a deregulated innate immune response in the colon. Yet, there is a possibility that stimulation of adaptive immunity could account for some of the responses to bovine milk OPN administration.
Bovine milk OPN decreases destructive capacity of neutrophils by activating macrophages

In the presence of OPN, macrophages infiltrating the injured mucosal tissues in early stages of acute inflammation help the neutrophils to combat invading pathogens, while concomitantly limiting the amount of damage by the neutrophils (reviewed in Sodek et al, 2006). We have shown that the absence of OPN expression in OPN-/− mice leads to increased destructive activity of neutrophils and improper differentiation of macrophages (Da Silva et al, 2006). However, we have also shown that the expression of OPN is not required for neutrophil destructive capacity (Koh et al, 2007). This increased neutrophil activity may be due to slow clearance by OPN-null macrophages (Hart et al, 2000), which display impaired migration (Zhu et al, 2004), suggesting that they may also have reduced phagocytic activity in vivo. Our most recent data show that the oral administration of 20 μg/ml of bovine milk OPN led to a reduction in neutrophil activity (Fig. 5.5 A) accompanied by an increase in TGF-β1 expression (Fig. 5.6). These results suggest that exogenous OPN may act by restoring macrophage activity.

Effective elimination of neutrophils is a prerequisite for resolution of the inflammatory response (Savill, 1997). One possibility in our colitis model is that exogenous OPN may contribute to the termination of inflammation by activating macrophages to take up apoptotic neutrophils, thereby leading to neutrophil clearance and the release of anti-inflammatory and reparative cytokines, such as TGF-β1 (Barker et al, 1999). Both in vitro and in vivo evidence suggest that secretion of TGF- β1 by macrophages can suppress proinflammatory signaling from Toll-like receptors, further stimulating tissue repair (Huynh et al, 2002; Lucas et al, 2003).
The attenuation of an inflammatory reaction coincides with the departure of macrophages through the lymphatics (Nathan, 2002; Lawrence et al, 2002; Savill, 1997). The tissue mononuclear cell population (macrophages and lymphocytes) returns to normal pre-inflammation density and phenotypes (Serhan et al, 2005). In support of this concept, our immunohistochemistry analysis of colon sections indicates that oral administration of milk OPN decreased the macrophage population during DSS-induced colitis (Fig 5.5 E). Consequently, it is possible that once apoptosis of leukocytes and resident cells is engaged (an early event), subsequent uptake of apoptotic cells would cause a switch in macrophage phenotype from activated or injurious to reparative or emigratory. Such a shift would also be expected to diminish the local production of proinflammatory cytokines, as we observed in our data set (Fig. 5.7). Exogenous milk OPN appeared to promote a more confined sequence of protective inflammatory responses, while limiting the intensity of the acute DSS-induced colitis.

TGF-β1, restitution of the mucosal epithelial barrier, and healing

The intestinal mucosal barrier is formed by a monolayer of tightly joined epithelial cells that most often becomes disrupted during colitis. It has been shown that the intestinal mucosa promotes epithelial restitution after mucosal injury through the increased production of bioactive TGF-β1 by the epithelial cells (Dignass and Podolsky, 1993) and by subepithelial myofibroblasts (McKaig et al, 1999). For example, epithelial monolayers pretreated with TGF-β1 completely abrogated the barrier-disrupting effect of the intestinal pathogen C. parvum oocysts (Roche et al 2000). In the present study of experimental colitis, the beneficial effects of administering 20 μg/ml milk OPN was
accompanied by a significant increase of TGF-β1 in both the OPN-/- and the WT mice (Fig. 5.6). The increase in TGF-β1 may have contributed to the maintenance of intestinal barrier integrity and intestinal healing in the mice that experienced less tissue destruction.

**OPN modification may be crucial for its attenuation of DSS-induced colitis**

Post-translational phosphorylation of OPN has a significant impact on the biological functions of this protein (reviewed in Kazanecki et al, 2007). Ashkar et al (2000) reported that phosphorylation of the N-terminal domain of OPN is required for RGD-β3-integrin recognition, and subsequent induction of interleukin-12 expression in murine macrophages. Similarly, phosphorylation of OPN is required for its stimulation of RGD-mediated spreading and activation of macrophages (Weber et al, 2002). Moreover, Al-Shami et al (2005) has shown that OPN induces trophoblastic cell migration, which is a process that depends on the level of phosphorylation of OPN. Notably, the recombinant OPN that we used in our study of experimental colitis, derived from the rat OPN sequence, did not promote the protective effects that were clearly associated with administration of bovine milk OPN, which is highly phosphorylated (Sorensen et al, 1995). While no detrimental effects were associated with the use of the r-OPN, its lack of protective effects may be explained by its freedom from post-translational modification. Alternatively, the different results for the two forms of OPN could have been due to their distinct phylogenetic source; divergence in primary sequence may have contributed to altered function. However, the functional domains of OPN are normally well conserved among species (Sodek et al, 2000), and it is more likely that such differences are due to the degree or position of phosphorylation.
Does the efficacy of exogenous milk OPN require expression of endogenous OPN?

In this study, we found that administration of milk OPN diminished the damaging effects of DSS more in the WT than in the OPN-/- mice. Though the OPN-/- mice, which express no endogenous OPN, experienced marginal protection, and therefore may have required a greater dose, we propose that intracellular OPN may be a critical cofactor for mediating the physiological responses required for the attenuation of DSS-induced colitis. Evidence to support this hypothesis comes from a number of studies that explored the relationship of CD44 and OPN. The intracellular form of OPN has a perimembranous distribution, and it co-localizes with CD44 and ezrin-radixin-moesin proteins in migrating embryonic fibroblasts, activated macrophages, and metastatic breast cancer cells (Zohar et al, 1998; Zohar 2000; Sodek et al, 2000). Impaired chemotaxis in OPN-/- macrophages (Zhu et al, 2004) and osteoclasts (Suzuki et al, 2002; Chellaiah et al, 2003a) has been associated with reduced cell surface expression of CD44. Moreover, whereas expression of OPN is evidently required for the recruitment of CD44 to the cell surface in macrophages and osteoclasts (Suzuki et al, 2002; Chellaiah et al, 2003a; Chellaiah et al, 2003b), this requirement is not evident in neutrophils (Alstergren et al, 2004). The ligation of CD44 by OPN mediates chemotaxis and adhesion of fibroblasts (Denhardt, 2001b) and the down-regulation of interleukin -10 ( IL)-10 expression in peritoneal macrophages (Ashkar et al, 2000). Furthermore, it has been suggested that the extracellular OPN can provide temporary CD44 or more substantial integrin attachment complexes that are required for the motility as well as the chemotactic functions of migrating cells (Sodek et al, 2000). Consistent with this concept, Lee et al (2007)
demonstrated that an OPN-CD44 interaction is able to increase integrin adhesion by activation of integrins via inside out signaling, resulting in increased cell survival. Therefore, it is conceivable that macrophages of OPN-/- mice may not be fully activated in the presence of exogenous OPN, which could impair the phagocytosis of neutrophils by the macrophages. Indeed, the administration of exogenous OPN led to only a minor, insignificant decrease in myeloperoxidase activity of tissues from the OPN-/- mice (Fig 5.5 A).

In summary, this study demonstrates that the administration of bovine milk OPN attenuates DSS acute colitis. The protective effect seems to depend on a post-translationally modified form of OPN, and it is diminished in mice that are genetically deficient in endogenous OPN expression. The mechanism by which exogenous OPN attenuates colitis may be due to 1) the modulation of the number of neutrophils and their destructive activities, 2) decreased proinflammatory cytokine release, and 3) restitution of the epithelial barrier through increased local expression of TGF-β1, all potentially affected through macrophage interactions.
Fig. 5.S1. BSA administration during DSS-induced colitis. A: In contrast to b-OPN (*, $P<0.05$, n=5), BSA failed to reduce DAI scores. B: In contrast to b-OPN (*, $P<0.05$, n=5), BSA failed to reduce the number of neutrophils, as shown by their levels in peripheral blood. C: Immunohistochemistry control. No staining was detected in colon tissue of WT DSS-treated mice in the absence of streptavidin-HRP complex. D: Colon tissue of DSS-treated WT mice that was not exposed to biotinylated BSA yielded only slight immunostaining in the epithelial cells of the mucosa. E: Immunostaining of colon sections of mice that were administered biotinylated BSA in drinking water yielded considerable labeling in the epithelial layer of the mucosa (see arrows).
**Fig. 5.S2.** Biotin and OPN immunostaining. **A:** Immunostaining of DSS-treated WT colon tissue that was not exposed to bovine biotinylated-OPN yielded slight immunostaining in the epithelial cells of the mucosa in the presence of streptavidin-HRP complex. **B:** Immunostaining of DSS-treated WT colon tissue that was not exposed to bovine biotinylated-OPN yielded abundant immunostaining, especially in the epithelial cells of the mucosa, when immunostained to detect OPN.
Fig. 5.S3. A, B, C, D: Hematoxylin and eosin staining of the colon sections equivalent to A, B, C, D figure 2 included in this manuscript.
Supplemental Figure 4

Fig. 5.S4. A, B, C, D: Hematoxylin and eosin staining of colon sections corresponding to tissue samples sectioned for Figure 5 A, B, C, and D of the main set of figures in the manuscript.
Supplemental Figure 5

A

Disease Activity Index

WT DSS
OPN-/- DSS

Day

B

% Weight Loss

WT DSS
OPN-/- DSS

Day

C

Water Consumption (ml)

WT DSS
OPN-/- DSS

D

Spleen/Body weight

WT DSS
OPN-/- DSS
**Fig. 5.S5.** Clinical analysis of WT and OPN-/- mice during DSS-induced colitis. **A:** DAI scores were greater in the DSS-treated OPN-/- than the DSS-treated WT mice on days 6 and 7, but the difference was not statistically significant. **B:** The weight loss in the DSS-treated WT mice was consistently greater than that in the DSS-treated OPN-/- mice, but statistically greater only on day 5. **C:** No significant difference was found in water consumption between the DSS-treated groups. **D:** OPN-/- DSS-treated mice exhibited significantly greater increased spleen weight than the DSS-treated WT mice (*, $P<0.05$, n=9).
Chapter 6: General discussion and future directions
Summary of major findings

The specific objective of this thesis, to investigate the functional and clinical significance of OPN in DSS-induced colitis, was primarily addressed in chapter 3. This study demonstrated that OPN-null mice are more susceptible to DSS-induced colitis than the DSS-treated WT control mice. The increased susceptibility of the OPN-/- mice to DSS was characterized by greater intestinal crypt destruction; high myeloperoxidase activity of infiltrating neutrophils; lack of differentiation of inflammatory cells such as lymphocytes subsets (CD4+, CD8+) and macrophage (F4/80); reduced production of certain cytokines, especially TNF-alpha; and non-programmed cell death of enterocytes. Collectively, these studies suggest the importance of OPN in maintaining the epithelial barrier (Fig. 6.1), as well as ensuring a normal inflammatory response, to protect against DSS-induced colitis. The inability of OPN-null mice to retain the integrity of the epithelial barrier (Fig. 6.1) reflects deficiencies in immune cell function, which is also likely to impact on the progression of other mucosal diseases. However, our results in the experimental colitis model contrast the decreased disease activity observed in the absence of OPN in some other inflammatory diseases (Miyazaki et al., 1995; Noiri et al., 1999; Chabas et al., 2001; Yumoto et al., 2002). This difference may reflect the outcome of deficiencies in immune cell function, which in colitis are required to combat a persistent bacterial exposure, due to the loss of barrier function. In addition, OPN also appears to be important for survival of certain cells that are essential for tissue repair. Consequently, OPN appeared to have a protective role in colitis and could, therefore, be a potentially useful target for developing therapeutic approaches to treat inflammatory bowel disease.
Figure 6.1. Increased permeability of the colon mucosa in the absence of OPN. FITC-dextran was administered orally by gavage to WT and OPN-/- mice on Day 7 of DSS treatment, and colon sections were examined by fluorescence microscopy. During colitis the OPN-/- mice exhibited decreased integrity of the mucosal epithelial barrier than the WT mice. This is observed by the increased submucosal infiltration of FITC-dextran in colon the of OPN-/- during colitis.
The murine DSS model that was used for these studies induced acute colitis, which is most likely caused by nonspecific injury, and is probably more dependent on innate immune cells, such as macrophages and neutrophils, than are inflammatory diseases due to specific etiology or that are chronic (Axelsson et al, 1996; Mahler et al, 1998; Dieleman et al, 1998). Accordingly, we suspected that the hyperactivity of neutrophils may explain the increased colon tissue destruction during experimental colitis in the absence of OPN. For this reason, the relevance of OPN expression in neutrophil function was determined (chapter 4). I found that while OPN may be important for the recruitment and migration of neutrophils, the expression of OPN by neutrophils is evidently not required for their destructive capabilities, as seen in the extent of DSS-induced tissue damage and the myeloperoxidase activity in OPN null mice (chapter 3).

Investigating the potential for exogenous OPN administration to attenuate the inflammatory destruction in DSS-induced colitis in the OPN-/- mice, was mainly addressed in chapter 5. I found that exogenous bovine milk OPN (20 µg/ml), administered for 8 days dissolved in the drinking water, ameliorated DSS-induced colitis in mice. It diminished signs of disease, presumably by reducing the levels of immune cells and pro-inflammatory mediators in the colon. Notably, opposite from what we hypothesized, the beneficial effect of exogenous OPN administration was greater in the WT mice than in the OPN -/- mice. I suggested that the different levels of protective effects observed in the OPN-/- and WT mice administered OPN may be explained by their differential expression of i-OPN, which seems to play an important role in immune cell activities necessary for the resolution of inflammation. This hypothesis is discussed in chapter 5. Based on the literature (chapter 1 and 2) and our findings (chapter 3, 4, and
5), I propose a mechanism by which OPN modulates the inflammatory immune responses during DSS-induced colitis.

**Hypothetical mechanism of action for OPN during DSS-induced colitis**

*Epithelial barrier destruction and macrophage and PMN recruitment in the absence of OPN*

We found that OPN is upregulated in the intestinal epithelial cells during DSS-induced colitis, and we concluded that it may be involved in the maintenance of barrier defenses by controlling the permeability of the mucosa (chapter 3; fig. 6.1, 6.2). Moreover, OPN may contribute to the expression of MHC-II and Toll receptors, which normally aid in antigen presentation and inflammatory signaling (Mowat, 2003; Basu and Fenton, 2004; Iwasaki and Medzhitov, 2004). In the OPN null mice, we found a greater number of ulcers in the epithelium, accompanied by evidence for increased non-programmed cell death. This suggests that rapid necrotic cell death, which can intensify inflammatory destruction and loss of epithelial barrier protection, may be a key event (fig. 6.2A). With the increased epithelial damage, there would be increased penetration of bacteria and release of IL-1, IL-8 and MCP-1, which recruits neutrophils and macrophages (fig. 6.2B) that could further damage the intestinal mucosa by releasing ROS.

*Increased mucosal destruction by neutrophils in OPN null mice*

Macrophages that are attracted and activated by cytokines secreted by neutrophils infiltrate the injured mucosal tissues in early stages of DSS-induced colitis. They would
be expected to help the neutrophils phagocytose invading pathogens, while coincidently modulating the extent of damage by neutrophils. In the absence of OPN there is increased destructive activity of neutrophils (Da Silva et al, 2006 and submitted; chapter 3 and 5). However, we have also found that the expression of OPN is not required for neutrophil activities that are potentially destructive (Koh et al, 2007; chapter 4). Conceivably, the increased neutrophil activity in OPN null mice may be due to delayed clearance mediated by OPN-null macrophages (Hart et al, 2000), which are known to display impaired migration (Zhu et al, 2004) and may also have reduced phagocytic activity. The concept that lack of i-OPN expression may impair phagocytosis by macrophages in OPN-/- mice may help explain why DSS-treated OPN-/- mice responded poorly in comparison with WT mice to exogenous OPN administration (discussion chapter 5).

**OPN activates macrophages during the resolution of inflammation**

It has been suggested that in the absence of OPN, macrophage differentiation and activities appear to be impaired (our data in chapter 3; Zhu et al, 2004; Marroquin et al, 2004). I found that administration of OPN to WT mice during experimental colitis promotes improvement of clinical signs of inflammation accompanied by a reduction in neutrophil activity, and a concomitant increase in TGF-β1 secretion in colon tissues (chapter 5). These findings suggest that the OPN modulatory effect on macrophages is a key mechanism that may explain OPN protective functions during DSS-induced colitis.

As discussed in chapter 5, exogenous OPN may activate macrophages which would then phagocytose apoptotic neutrophils. Since macrophages that ingest dying neutrophils are known to release more TGF-β1 (Barker et al, 1999), this could further
stimulate tissue repair (fig. 6.2B). Consequently, I suggest that once apoptotic neutrophils are engaged by macrophages, a switch in macrophage phenotype from activated or injurious to reparative or emigratory would occur. Furthermore, according to this hypothesis, a reduction of pro-inflammatory mediators would also result, as was demonstrated (in chapter 5), and macrophages would leave the local mucosal site through the lymphatics (fig. 6.2C). Indeed, there was a decrease in macrophage population levels in colon tissues of WT mice that had been exposed to exogenous OPN (chapter 5).

**OPN induction of mucosal tissue repair**

The attenuation of experimental colitis, accompanied by increased levels of TGF-β1 in the presence of OPN, suggests that colonic tissue repair is fostered by the activity of OPN. The intestinal mucosal barrier is formed by a monolayer of epithelial cells that is usually disrupted during colitis. Studies have shown restitution of the epithelial barrier in the presence of TGF-β1, which induces epithelial cell migration and thereby an increase of cell surface area and tissue integrity (Roche et al 2000; Neunlist et al 2007). Therefore, OPN may contribute to the restitution of the epithelial barrier (fig. 6.2D). In addition, the increased release of TGF-β1, together with the exogenous bovine OPN, may down-regulate fibroblast apoptosis and increase matrix deposition (fig. 6.2E) (Desmoulière, 1995; Jelaska and Korn, 2000; Zohar et al, 2004). Consequently, with appropriate macrophage function, the immune defense for the proper resolution of the inflammatory response would be restored in the presence of OPN, leading to decreased overall destruction of colon tissues.
Figure 6.2. OPN promotes mucosal protection during DSS-induced colitis. A. Increased neutrophil activity due to slow clearance by OPN-null macrophages, and non-programmed cell death in the absence of OPN. B. OPN may activate macrophages which would take up apoptotic neutrophils, and macrophages ingesting dying neutrophils would release TGF-β1. C. Switch in macrophage phenotype from activated or injurious to reparative or emigratory. Macrophages leave through the lymphatics. D. Restitution of the epithelial barrier in the presence of TGF-β1. E. Increased release of TGF-β1 together with OPN downregulate fibroblast apoptosis and increase matrix deposition.
A current, more logistic interpretation of my data

It was previously proposed (Da Silva et al, 2006; chapter 3) that the exacerbated inflammatory response and increased colon mucosal damage in the absence of OPN was due to a combination of impaired macrophage function and lack of proper activation of the adaptive immune response (fig 6.3). The impaired differentiation of T cells and mobilization of lymphocytes from lymph nodes, combined with improper macrophage functions and decreased levels of pro-inflammatory cytokines, resulted in persistence of neutrophil activity leading to extensive intestinal mucosal damage. However, from our studies on the effect of administration of milk OPN to DSS-mice (chapter 5), I concluded that the beneficial effects of exogenous OPN was mainly due to a modulation of the innate immune response. This interpretation was based on my recent results and on other published studies, which suggests that the involvement of the adaptive immune response directly in the DSS acute colitis model may be unlikely.

It has been described different DSS model protocols that would lead to either acute or chronic colitis in mice (see chapter 1). Many studies have excluded the requirement for functional T and B lymphocytes in the acute DSS colitis model (Dielman et al, 1994; Axelsson et al, 1996; Mahler et al, 1998). Moreover, numerous attempts were made in this study to detect CD3 antigen-positive T cells by using immunohistochemistry in the DSS-treated mice colon tissues, but none was detected in either OPN-/− or WT mice. Since this antigen is known to be present in T cells and involved in the pathogenesis of chronic colitis, it is possible that the absence of CD3+ lymphocytes may occur in this acute colitis model.
In contrast, CD3+ T cells are expected to be involved in the pathogenesis of chronic colitis. Indeed, CD3+CD4+ T cells are in increased levels in the colon lamina propria during chronic colitis in mice (Watanabe et al, 1998), and a recent study used an agonistic anti-CD3 antibody to successfully ameliorate chronic DSS induced colitis (Melgar et al, 2008). Therefore, even though OPN may be important for proper differentiation of T cells from the spleen, as my earlier data suggested (Da Silva et al, 2006; chapter 3; fig. 6.3), my recent results and published literature suggest that the activation of the adaptive immune response is not significantly involved in the inflammatory process seen in the DSS acute colitis model. This concept was developed further in the interpretation of the OPN-rescue studies described in chapter 5.

**Comparison of recent studies on OPN function during DSS-induced colitis**

It has been suggested that OPN may function as either a pro-inflammatory or an anti-inflammatory cytokine, which would depend on the stimulus and the type of host immune response involved in the site-specific inflammatory process (Wang & Denhardt, 2008). Similarly, IL-22, known to be associated with the innate and adaptive immune response, appears to be a dual-natured cytokine. It is involved in development of dermal inflammation (Zheng et al, 2007), and alternatively, it can provide protection during liver inflammation (Zenewicz et al, 2007) and DSS-induced colitis by possibly modulating the innate immune response. My studies found that during experimental colitis in C57BL/6 mice (5% DSS for 7 days), the absence of OPN caused increased colon tissue destruction (Da Silva, 2006). In the Black Swiss mice strain, using 3.5% DSS, OPN appeared to attenuate experimental colitis (Zhong et al, 2006). Perhaps, the acute colitis developed in
Figure 6.3. OPN involvement in the innate and adaptive immune response during DSS-induced colitis.

A. Upregulated OPN in the intestinal epithelial cells is involved in the barrier defence process. B. Epithelial damage, penetration of bacteria, release of chemokines which recruit neutrophils and macrophages. C. In the OPN−/−, increased destructive activity of neutrophils due to slow clearance by OPN-null macrophages, which appear to be not properly differentiated. D. OPN is thought to be involved in the differentiation, maturation, and survival of APC, which control T cell differentiation. Improper presentation will lead to lower number of defending T lymphocytes and dysregulated inflammatory response. E. OPN contributes to the activation of naïve T cells resulting in the differentiation of T-helper and cytotoxic T cells. In the absence of OPN, differentiation of CD4 and CD8 lymphocytes is impaired. F. In the OPN−/−, greater number of ulcers in the epithelium accompanied by increased non-programmed cell death that can intensify inflammatory destruction.
the Black Swiss mice strain is less severe than the colitis developed in the C57BL/6 mice, for which innate immunity may be playing a more prominent role. Indeed, in agreement with our study, a more recent study that used the C57BL/6 mice showed increased tissue destruction in the absence of OPN during acute DSS-induced colitis (Heilmann et al, 2008). In contrast, in chronic colitis, in which the initial DSS application was followed by normal drinking water for 10 days and repeated four times, they showed that the absence of OPN attenuated experimental colitis (Heilmann et al, 2008). It has been suggested that during chronic colitis, OPN induces the T-cell driven inflammation since it has been shown that OPN induces Th1 polarization (Ashkar et al, 2000; Chabas et al, 2001; Jansson et al, 2002; O’Regan, 2003). I would speculate that this could also happen in a less severe case of acute colitis, as described in the Black Swiss mice (Zhong et al, 2006) in which a less concentrated dose of DSS (3.5%) and a longer treatment period (10 days) were used. Therefore, the possibility that OPN is a dual function cytokine during intestinal inflammation may explain differences in results shown in recent reports. In severe cases of acute inflammation, as seen in our studies, OPN apparently activates innate immunity, reduces tissue damage by possibly activating macrophages, and initiates mucosal repair. During less severe acute colitis and chronic inflammation, induced by less aggressive DSS treatment protocols, it evidently promotes the Th1 response, which enhances inflammation.
Future directions

*The role of exogenous bovine milk OPN in remodelling and repair during DSS-induced colitis*

My data clearly show attenuation of DSS-induced colitis with the administration of exogenous OPN to WT mice. This suggests that OPN may have an important function in the resolution of the inflammatory process and in the repair of mucosal tissues. The amelioration of DSS-induced colitis was accompanied by increased TGF-β1 in colon tissues, which suggests mucosal healing due to the presence of exogenous OPN. TGF-β1 has been shown to have a major role in intestinal epithelial restitution (Dignass and Podolsky, 1993), promoting wound healing by the deposition of extracellular matrix proteins, such as collagen I and III (Border and Noble, 1994; Kenyon et al, 2003), and the suppression of MMPs (Overall et al, 1991). Furthermore, TGF-β1 may induce differentiation of fibroblasts to myofibroblasts, and inhibits apoptosis of the myofibroblasts, which may cause extended survival of this population and thus greater collagen deposition (Zhang and Phan, 1999). The formation of repair tissue is dependent upon the generation and persistence of myofibroblasts. In addition to their potential regulation of epithelial restitution (McKaig et al, 1999), subepithelial intestinal myofibroblasts express a number of extracellular matrix proteins and enzymes that may influence epithelial cell function (Mahida et al, 1997).

Tissue degradation can be identified through the detection of MMPs and denatured collagen. In particular, deposition of collagens I and III, and proliferation of epithelial cells are indicative of intestinal repair and restitution of the epithelial barrier.
Previous studies have shown that the presence of denatured collagen is indicative of collagen remodelling (Rucklidge et al., 1986) and staining for denatured collagen is strong in tissues with a rapid collagen turnover (Salonen et al., 1990). Therefore, it would be logical to determine whether OPN administration affects the components of matrix remodeling and repair by the study of epithelial cells proliferation, metalloproteinase expression, presence of myofibroblasts, and deposition of collagen in DSS-induced colitis.

Several studies have shown that MMP-2, 3, 9 are over-expressed in IBD inflamed colonic mucosa of IBD patients and are associated with disease activity (Bailey et al, 1994; Stallmach et al, 2000; Von Lampe et al, 2000). Castaneda et al (2005) have shown that DSS-induced colitis is attenuated in MMP-9-/- mice. In contrast, MMP-2 has been reported to serve a protective role in development of DSS-induced colitis (Garg et al, 2006). However, DSS-induced colitis was attenuated in MMP-2/MMP-9 double knockout mice. Thus, it would be of considerable interest to determine whether MMP-2, 3 and 9 levels, (latent, activated and transcripts) are modulated by administration of milk OPN. My MMP analysis using zymography have shown both precursor and activated forms of matrix metalloproteinase-2 (MMP-2) in the DSS-treated mice (Da Silva et al, 2006; chapter 3). Interestingly, MMP-9 was not detected (Da Silva et al, 2006; chapter 3). It was indicated that the gelatinase activity was not from neutrophils but may be derived from macrophages or mesenchymal cells, which would explain the absence of MMP-9 in colitis gut tissues (Da Silva et al, 2006; discussion chapter 3). The level of activated MMP-2 was approximately 2-fold lower in the colons from the OPN-null mice than the WT mice (Da Silva et al, 2006; chapter 3). The greater activation of MMP-2 in the DSS-
treated WT animals may be associated with tissue repair, given the selective increase in
the expression of this MMP by the profibrotic cytokine TGF-β that has been reported in
the literature (Overall et al, 1991).

Using the acute and chronic DSS colitis models in C57BL/6 mice, there is
extensive deposition of collagen in the mucosa and submucosa of inflamed colons
(Melgar et al 2005). My preliminary data using immunostaining showed collagen I levels
greater in the WT mice than the OPN-/- mice sections (Fig. 6.4A). Furthermore the level
of staining appears to be increased in the DSS-treated mice. Since OPN promotes tissue
repair and oral administration of milk OPN induced increased levels of TGF-β1 (chapter
5), I would also expect increased deposition of collagen I, and especially, collagen III in
both OPN-treated WT and OPN-/- mice. These collagen levels should further increase
following the withdrawal of DSS. In addition, using image analysis, I performed
morphometric measurements of the extension of the mucosa and the submucosa of colon
sections from the lumen into the gut wall. There was a great increase in the extension of
both mucosa and submucosa in the WT DSS-treated mouse tissues that was not observed
in the OPN-/- DSS-treated mouse colon (Fig. 6.4B). This thickening in the mucosa and
submucosa likely reflects tissue repair. As before, it would be of interest to determine
whether the effects of oral administration of OPN enhanced matrix deposition during the
time course of DSS-administration and the outcome one week after cessation of DSS.
Fig. 6.4. OPN promotes matrix deposition in DSS-induced colitis. A. Collagen I immunostaining seems to be increased in the DSS-treated mice, greater in the submucosa of WT mice than the OPN-/- mice sections. B. Morphometric measurements showed greater increase in the extension of both mucosa and submucosa in the WT DSS-treated mouse tissues, not seen in the OPN-/- DSS-treated mouse colon.
The impact of diverse forms of OPN during DSS-induced colitis

OPN may be present in a number of distinct isoforms that differ in post-translational modifications (see chapter 1), which may alter the biological functions of this protein (reviewed in Kazanecki et al, 2007). It was of interest that oral administration of recombinant OPN failed to attenuate DSS-induced colitis when compared with the administration of bovine milk OPN (chapter 5). These data suggest that the attenuation of mouse experimental colitis seen in our studies may be due to phosphorylation or some other modification of the bovine milk OPN (Sørensen et al, 1995). Levels and specific sites of OPN phosphorylation between the various tissue-specific forms are likely important modulators of its function. The phosphorylation sites from bovine milk OPN have been mapped (Sorensen, 1995). Sorensen et al (1995) have identified twenty-seven phosphorylated serines in bovine milk OPN. In chapter 1 of this thesis, several studies are described in which phosphorylation of OPN seems to be critical to the induction of specific immune cell activities, such as spreading and activation of macrophages (Weber et al, 2002). Therefore, due to significant functional implications of OPN phosphorylations in cell activities, it would be logical to determine whether it is only the fully phosphorylated isoform of OPN which is active in colitis or whether partially phosphorylated OPN such as recombinant OPN treated with protein kinase CK2 (Salih et al, 1996; Saad et al, 2008) would also induce amelioration of DSS-induced colitis. These experiments would be relatively easy to perform since rOPN is easily expressed and purified (Zhang et al, 2007), and protocols for CK2 treatment and phosphate content and localization by mass spectrometry (Keykhosravani et al, 2005; Saad et al, 2008) have been described.
OPN modulates inflammation through a variety of motifs that recognize several different receptors (integrins, CD44) (see chapter 2). For example, the amino-terminal sequence of OPN induces chemotactic and cytokine responses of macrophages through the CD44 receptor (Ashkar et al, 2000; Weber et al, 1996), while up-regulation of IL-12 in macrophages is mediated by RGD signaling through the \( \alpha v \beta 3 \) integrins (Ashkar et al, 2000; Weber et al, 2002). Thrombin digestion of OPN un masks a cryptic integrin binding site that also elicits cell signaling through CD44 receptors (reviewed Sodek et al, 2000; Sodek et al, 2006; chapter 2). Thus a specific sequence in OPN may be all that is required to initiate wound repair responses. The utilization of a specific segment of OPN that optimally attenuates DSS-induced colitis, may allow for the reduction of the deleterious effects of administering full-length OPN. To determine the specific domains of OPN important for attenuation of DSS-induced colitis, OPN-derived peptides (e.g. thrombin generated N-terminal and C-terminal; or CK2-treated rOPN peptides) could be individually tested. Specifically, the efficacy of these peptides could be first tested by *in vitro* assays, such as macrophage chemotaxis (Zhu et al, 2004) or alternatively the peptides could be tested for their ability to modulate expression of cytokines by OPN-/- macrophages stimulated with LPS. Ultimately, the peptides would be tested in the *in vivo* DSS-induced colitis model described in chapter 3 and 5.

As discussed in the introduction of this thesis, biological therapy utilizing infliximab, which targets TNF-\( \alpha \), has been used successfully in the treatment of some forms of IBD. However there are a number of significant side effects found with these therapies, as well as concerns with the effect of blocking fundamental functions of these cytokines during an inflammatory process. For example the immune suppressive action
of infliximab could result in serious infections (reviewed in Stokkers and Hommes, 2004). My studies provide evidence to suggest an alternate biological therapy that may be more natural and with fewer potential side effects. Therefore, these studies may potentially contribute to a novel therapeutic approach for IBD.
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