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EFFECTS OF OXIDATIVE STRESS ON ANTIOXIDANT DEFENSE AND INFLAMMATORY RESPONSE IN INTESTINAL EPITHELIAL CELLS

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ABSTRACT

To characterize the role of intestinal epithelial cells in mucosal host defense, we have examined endogenous antioxidant reactivity and inflammatory response in Caco-2 cell line exposed to oxidative stress. When differentiated Caco-2 cells were incubated with iron/ascorbate for 1 to 24 hours, they exhibited increased MDA levels and decreased polyunsaturated fatty acid proportion in favor of saturated fatty acids. These alterations were accompanied with small alterations in membrane fluidity and permeability. The oxidative stress did not induce changes in the antioxidant enzyme activity of superoxide dismutase, catalase, glutathione peroxidase and glutathione transferase, or in cellular glutathione content. However, iron/ascorbate-mediated lipid peroxidation promoted IκB degradation and NF-κB activation, as well as gave rise to interleukin-8, COX-2 and ICAM-1. These results support the importance of oxidant/antioxidant balance in the epithelial cell inflammatory response.

Key Words

Caco-2 ; oxidative stress ; cell integrity ; endogenous antioxidants ; NF-κB ; ICAM-1 ; COX-2 ; IL-8.

SOMMAIRE

Afin de caractériser le rôle des cellules intestinales épithéliales dans les mécanismes de défense de la muqueuse intestinale, nous avons vérifié la réponse antioxydante endogène et inflammatoire au stress oxydatif dans le modèle intestinal Caco-2. Une fois différenciées, les cellules Caco-2 en monocouche ont été incubées avec le complexe fer/ascorbate durant des périodes de 1 à 24 heures. Elles affichèrent une augmentation des taux de malondialdehyde et une diminution de la proportion des acides gras polyinsaturés en faveur des acides gras saturés. Ces altérations furent accompagnées de changements mineurs dans la fluidité membranaire et la perméabilité cellulaire. De plus, la présence de stress oxydatif a été incapable d'induire des changements dans l'activité des enzymes superoxyde dismutase, catalase, glutathion peroxidase et glutathion transferase, ni dans les taux de glutathion cellulaire. Cependant, la péroxydation induite par la présence du fer/ascorbate a déclenché la dégradation du IκB et l'activation du facteur de transcription NF-κB, de même qu'une augmentation de IL-8, ICAM-1 et COX-2. Ces résultats soulignent l'importance de l'équilibre oxydant/antioxydant dans la réponse inflammatoire des cellules intestinales épithéliales.

Mots clés

Caco-2 ; stress oxydatif ; integrité cellulaire ; antioxydants endogènes ; NF-κB ; ICAM-1 ; COX-2 ; IL-8.

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γ-GCS : gamma glutamyl cysteine synthetase

γ-GT : gamma glutamyl transferase

μM : micromolar

5-ASA : 5-aminosalicylic acid

6-MP : 6-mercaptopurine

8-OHdG : 8-hydroxy-2-deoxyguanosine

AIDS : acquired immunodeficiency syndrome

AZA : azathiopurine

BHA : butylated hydroxyanisole

BHT : butylated hydroxytoluene

Ca : calcium

cAMP : cyclic adenosine monophosphate

CD : Crohn's disease

$CD4^+$ or $CD\ 8^+$: cluster of differentiation 4^+ or 8^+

CDISM : Crohn's disease intestinal smooth muscle

COX : cyclooxygenase

CSF : colony-stimulating factor

Cu : copper

DIPS : disopropyl salicylate

DMSO : dimethyl sulfoxide

DNA : deoxyribonucleic acid

ENA-78 : epithelial-derived neutrophil activating-78

FA : fatty acid

FAE : follicle-associated epithelium

Fe : Iron

fMLP : formyl-methionyl-leucyl-phenylalanine

gr : gram

GALT : gut-associated lymphoid tissue

GI : gastrointestinal

GRO : growth related oncogene

GSH : reduced glutathione

GSH-Px : glutathione peroxidase

GSSG : oxidized glutathione

GSSG-Red : glutathione reductase

GST : glutathione transferase

H_2O_2 : hydrogen peroxide

HO· : hydroxyl radical

HOCl : hypochlorous acid

HQ· : semiquinone radical

hr : hour

IκB : inhibitor protein kappa B

IBD : inflammatory bowel disease

ICAM : intracellular adhesion molecule

IEC : intestinal epithelial cell

IEL : intraepithelial layer

IFN : interferon

Ig : immunoglobulin

IKK : Inhibitory protein kappa B kinase

IL : interleukin

IL-1Ra : interleukin-1 receptor antagonist

iNOS : inducible nitric oxide synthase

IP-10 : interferon-inducible protein-10

ISM : intestinal smooth muscle

kDa : kilodalton

Kg : kilogram

L· : lipid carbon-centred radical

LO· : lipid alkoxyl radical

LOO· : lipid peroxyl radical

LPMC : lamina propria monocytic cells

LPS : lipopolysaccharide

LRR : leucine rich repeats

MCP : monocyte chemotactic peptide

M-CSF : macrophage colony-stimulating factor

MDA : malondialdehyde

mg : milligram

MG-CSF : macrophage-granulocyte colony-stimulating factor

MHC : major histocompatibility complex

min : minute

MIP : macrophage inflammatory protein

ml : millilitre

mM : millimolar

mRNA : messenger ribonulceic acid

n-3 : omega 3

n-6 : omega 6

NAC : N-acetyl-L-cysteine

NADPH : nicotinamide-adenine-dinucleotide-phosphate

NDGA : nordihydroguaiaric acid

NF-κB : nuclear factor kappa B

NHISM : normal human intestinal smooth muscle

NIK : nuclear factor kappa B inducing kinase

NK : natural killer cells

NO : nitric oxide

NOS : nitric oxide synthase

O_2 : singlet oxygen

$O_2^{\cdot-}$: superoxide anion

PBMC : peripheral blood monocytic cells

PDTC : pyrrolidine dithiocarbamate

PG : prostaglandin

PUFA : polyunsaturated fatty acid

RANTES : regulated upon activation normal T cell expressed and secreted

RNA : ribonucleic acid

ROM : reactive oxygen metabolite

ROO· : peroxyl radical

ROS : reactive oxygen species

SCFA : short chain fatty acid

SEM : standard error of the mean

SOD : superoxide dismutase

TCR : T cell receptor

TGF : transforming growth factor

Th : T helper cells

THP : tetra-hydroxy-papaveroline

TNF : tumor necrosis factor

TPN : total parenteral nutrition

UC : ulcerative colitis

UV : ultraviolet

VCAM : vascular cell adhesion molecule

Zn : zinc

To Didier...

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Chapter 1

Introduction to Crohn's Disease

1.1 DESCRIPTION OF CD

It has been nearly 70 years since the first description of regional ileitis, a disorder of the gastrointestinal tract which later became known as Crohn's disease (CD), in homage to the scientist who first described the condition [1]. CD, along with ulcerative colitis (UC), a similar albeit distinct condition, constitute human inflammatory bowel disease, chronic conditions which affect countless individuals throughout the world. These disorders affect the digestive system and cause the intestinal mucosa to become inflamed, resulting in clinical symptoms. CD can affect any part of the digestive tract, from the mouth to the anus, and typically expresses itself with patches of inflammation separated by healthy tissue. The inflammation can span through every layer of affected tissue, and there is no known cure for the disease. Drugs or surgery can only help relieve symptoms. Most patients experience periods of remission and flare-ups of the disease, and require medication, hospitalization or surgery. Affected individuals are most often diagnosed with CD between the ages of 15-25 or 45-55. More importantly, the exact cause of CD remains unknown. It is thought to be a multi-etiological disorder, with several genetic and environmental predisposing factors. Canada is believed to have one of the highest rates of CD in the world, and the condition poses quite a burden on our health care system.

1.1.1 EPIDEMIOLOGY OF CD

One of the most notable facts about CD is that it is not distributed evenly in all populations throughout the world. Its incidence varies with age, gender, geography, socio-economic status, ethnicity and time. The incidence rates for CD range between 1 and 10 per 100 000 population overall, and this can be even higher in the paediatric age group [2][3]. Age-specific incidence rates for CD exhibit a bimodal distribution, with the largest peak occurring early in the third decade of life, and a smaller, second peak in the sixth decade of life. This may reflect varying environmental influences for different age groups,

although no conclusive evidence has yet been found [4][5]. Concerning gender differences, certain studies report a higher incidence (20%) in women, which implies that hormonal and lifestyle factors are likely to play a yet unidentified role [6][7]. Geographic variability of CD is striking, with the highest incidence rates being in Scandinavia, Great Britain, Northern Europe and North America. Central and Southern Europe, South Africa and Australia exhibit lower rates, while Asia, Africa, Latin America and South Africa have the lowest rates [8][9]. Moreover, there seems to be a North to South gradient in Europe and North America, with higher latitudes having a higher incidence of CD [10]. Since populations in northern climes are exposed to less sunlight, they tend to have lower levels of calcium and vitamin D, which could act as protective agents, possibly rendering them more at risk for CD.

Another remarkable point about CD is that, within the same geographic region, its incidence varies with social class and the level of industrialization. Most studies report a higher incidence of CD in people of higher socioeconomic status and living in industrialized or urbanized areas. This implies that environmental factors such as diet, pollutants and occupational activity have a preponderant role when it comes to CD susceptibility [8][9][11][12]. In addition, CD exhibits ethnic variability, with whites being more commonly affected than non-whites and Jews more than non-Jews. Even among Jewish populations in Israel, Ashkenazi Jews have higher rates of CD than those of Sephardic origin. This, once again, indicates that occupational and dietary factors may be at the root of differences in disease incidence. Ethnic differences in disease expression also suggest that there is a genetic predisposition for CD in certain populations [13][14]. With regard to variability over time, the incidence rates for CD have risen dramatically over the past 30 years. In the Western world, the most rapid increase occurred during the period of 1965-1980, which, after a slower increase ensued, reached a plateau in most countries and even decreased in some regions. This rise in CD may reflect changes in diagnostic procedures, as well as environmental, infectious, industrial and lifestyle factors, over time [2][7][15][16].

1.1.2 CLINICAL FEATURES OF CD

CD can manifest itself with both gastrointestinal and extra-intestinal symptoms. While the digestive symptoms are almost invariably present at diagnosis, extra-intestinal manifestations may be observed in up to 30% of patients, and for some, may even be the first or sole sign of CD. There may be a prolonged interval (up to 5 years) between onset of symptoms and a confirmed diagnosis of CD, particularly in children. That is mostly because many common symptoms, such as fatigue, weight loss, abdominal pain and diarrhea, are non-specific and are not necessarily investigated until they have been present for quite some time [17][18].

Among the gastrointestinal symptoms, abdominal pain and diarrhea are the most common and are present in the majority of patients. The abdominal pain is most often located in the right lower quadrant, which signifies terminal ileal involvement. However, epigastric discomfort can be observed in case of gastro-duodenal CD and periumbilical pain may be observed in the event of colonic or diffuse small bowel involvement. As for the diarrhea, it may be variable in severity, ranging from soft stools to marked watery diarrhea. Blood may also be present in the stools, mostly in the case of colonic involvement. Anorexia, nausea and vomiting are not uncommon. Perianal disease can also be present in some instances, with abscesses, fissures and fistulae. It is also noteworthy that the anatomic distribution of CD in the GI tract is not similar in the adult and paediatric population; whereas children present a higher proportion of small bowel involvement, adults are more likely to exhibit colonic disease [19].

With regards to extra-intestinal symptoms, fatigue, fever, and weight loss are noted in a majority of patients. Besides these systemic manifestations, the most frequent target organs for extra-intestinal manifestations are the skin, joints, eyes, liver, and bones [17]. For instance, patients with CD can suffer from eryhtema nosodum, pyoderma gangrenosum and aphtous stomatitis. Peripheral and axial joint involvement can also be observed in the form of arthritis or ankylosing spondylitis. As well, a number of patients with arthritis also develop

ocular inflammation. Regarding hepatic involvement, abnormal serum aminotransferases can be noted during the course of the disease, and in very extreme cases (less than 1% of patients) certain complications such as sclerosing cholangitis may lead to cirrhosis or even liver failure. Diminished bone density has also been frequently reported in CD patients, both in adults and children. Poor diet, inadequate calcium intake, excessive cytokine production and corticosteroid administration are just a few of the factors that affect bone density in CD [20].

Among the most notable features of CD are the nutritional deficiencies. Historically, malnutrition has been associated with CD ever since the initial description of the disease. The cause of malnutrition is multifactorial. Inadequate caloric intake is by far the most important cause. Malabsorption, increased nutrient losses, increased requirements and drug-nutrient interactions can also contribute to malnutrition. Inadequate intake is mostly due to anorexia or fear of abdominal pain or diarrhea, which are often associated with eating. Malabsorption can be due to the loss of absorptive intestinal surface, short bowel syndrome, or rapid intestinal transit. As for the increased losses, they are usually associated with exudation from the mucosa, which can lead to protein, electrolyte and fatty acid losses. Moreover, the active inflammation, increased mucosal cell turnover, and infection that are often present in CD can lead to increased nutritional requirements. Finally, several drugs such as sulfasalazine and antibiotics have important effects on nutrient absorption [21][22]. In light of all the aforementioned mechanisms, it is not surprising that an alarming proportion of patients suffer from nutritional deficiencies. Weight loss is a very common feature of CD and can affect 65-75% of patients. Between 25 and 80% of patients have hypoalbuminemia, which may be due to decreased protein absorption, intestinal protein loss, or increased catabolism. As for decreased fat absorption, it may contribute to deficiencies in fat-soluble vitamins, as well as in calcium, magnesium, and zinc. Anemia is also common in CD patients, as are deficiencies in iron, folate, and vitamin B12 [23]. Growth retardation is another common feature of CD, with about 40% of pediatric cases suffering from delayed linear growth. In some patients, a decrease in growth velocity may

precede gastrointestinal symptoms by months or even years. The contributing factors include various deficiencies and daily corticosteroid administration.

Given that CD patients are particularly susceptible to these deficiencies, nutritional assessment and monitoring is of critical importance in order to correct for any insufficiencies that might aggravate the patient's condition.

1.2 ETIOLOGY OF CD

As noted above, CD is a multifactorial disease, with no single known factor that predisposes to the condition. Rather, there is a myriad of factors, the interactions of which suggest that a certain individual may be at a higher risk for CD than another. Among these factors, the most prominent are genetic susceptibility, dietary factors, microbial infections, smoking habits, and immune dysfunction. This chapter will focus on the first four factors, while immune dysfunction will be explored in detail in the following chapters.

1.2.1 GENETIC FACTORS AND CD

The first case of related patients with CD was reported by Crohn and colleagues in their 1932 article, describing a 14-year old boy and his sister, both of whom were affected [1]. That was the first indication that there was a familial or genetic component to CD. With time, scientific research has led to a large body of evidence that susceptibility to CD is in large part genetic. Twin studies, familial risk data, and segregation analyses have all demonstrated that there is a strong genetic component to IBD, particularly in CD.

Twin studies are very useful in determining the extent to which both genetics and environment influence disease susceptibility [24][25][26]. Several studies have examined the concordance for CD in monozygotic and dizygotic twins of affected individuals. Various results reported a concordance of 50-65% in monozygotic as compared to 3-8% in dizygotic twins. This indicates that CD is not a purely genetic disease, since the concordance rate was always far less than 100% in monozygotes, despite sharing the exact same genetic material. These results thus provide evidence for the key environmental component of CD. At the same time, these results indicate that CD is not purely an environmental condition; if it were, then genes would not make a difference and the rates for monozygotes and dizygotes would be equal.

Studies covering familial risk data have added to the conclusive evidence of the important genetic component to the risk of CD. Familial clustering of

CD is common, and many studies have demonstrated an increased prevalence of CD among relatives of patients suffering from CD [27]. First-degree relatives are at greatest risk, particularly siblings; more distant relatives also display an increased disease prevalence. In addition, several family studies of the clinical patterns of CD have demonstrated high degrees of concordance regarding disease location, type, and age at diagnosis for relatives of affected individuals [28][29]. Another interesting feature of CD is 'genetic anticipation', a phenomenon in which the age of onset of an inherited disease decreases with successive generations [19][30][31]. Several studies have found that children are diagnosed an average of 10 to 17 years younger than their affected parents. The explanations behind genetic anticipation remain unclear.

Since the technological tools permitted it, scientists have been running segregation analyses in order to better understand the genetic factors behind CD. The data suggest that CD is associated with a genetic defect that is inherited in an autosomal recessive pattern. However, more than one gene is involved. CD thus fits into the category of a complex genetic disorder in that inheritance does not follow simple Mendelian models [32][33][34]. Current research into the genetics of CD can be grouped into two types of studies: genome-wide linkage analyses, and candidate gene studies.

In genome-wide linkage analyses, identification of specific regions that are shared more often by affected individuals than expected from Mendelian laws provides the location of the susceptibility loci. Initial genome-wide linkage studies by Hugot and colleagues and Satsangi and co-workers identified two susceptibility loci, IBD1 on chromosome 16 and IBD2 on chromosome 12, which are linked significantly to CD [32][34]. Subsequent research has sometimes confirmed both loci, whereas other studies did not provide support for either. Several other loci have been identified by genome-wide approaches. Satsangi and colleagues have also identified two regions, on chromosomes 3 and 7, while Cho and colleagues added 3 other loci, on chromosomes 1, 3, and 4 [34][35]. A Canadian genome-wide search recently revealed two new loci, on chromosomes 5 and 19 [36]. Other loci that showed some linkage to CD include chromosomes 6, 10, 12, 14, and 17. Several of these loci will probably be discarded, whereas

additional loci may be localized before the genetic picture to CD susceptibility can be revealed. In the mean time, genome scans have proven that CD has many susceptibility loci, that CD and UC are distinct disorders both having a genetic basis, and that some of the loci are interactive.

The candidate gene approach, on the other hand, examines genes that, by the various functions of proteins they produce, may be involved in CD. Such genes include the HLA class 2 genes. Positive associations have been found between CD and phenotypes DR7, DRB3 and DQ4. A significant association with the DRB1*01 allele was also found [37][38]. However, most studies reporting the association between CD and major histocompatibility complex (MHC) genes have yielded inconsistent, if not conflicting results. Studies on tumour necrosis factor (TNF) genes, also located within the MHC region, have given discordant results as well [39][40]. Genes encoding other cytokines and cytokine antagonists have also been of great interest; the genes for the interleukin-1 (IL-1) antagonist, IL-1Ra, and for IL-2 and IL-10 have been studied, although no association between these genes and CD has been reported yet [41][42][43]. Other genes being investigated include intercellular adhesion molecule (ICAM)-1, T cell receptor, mucin, motilin, vitamin D receptor, to name a but a few; and the list is still growing. Since complex mechanisms involving the immune system, epithelial function, and host response to microorganisms have been proposed as being involved in CD, a large number of genes may be considered as good candidates.

Most recently, and most prominently, three independent groups have reported that mutations in the NOD2 gene on chromosome 16 are associated with CD, thus confirming previous findings on IBD1 [44][45][46]. NOD2 is a member of the NOD1/APAF1 gene family, and has been identified and mapped to chromosome 16q12. This gene family has a role in inflammatory responses to bacterial triggers. NOD2 in particular responds to bacterial factors such as lipopolysaccharide (LPS). NOD2 expression is limited to monocytes, and it comprises an amino-terminal effector domain, a nucleotide-binding domain, and leucine-rich repeat (LRR) regions. This LRR domain has binding activity for bacterial LPS, and subsequently activates the NF-κB signaling

pathway [47]. Deletion of the entire LRR region leads to enhanced NF-κB stimulation [48]. Groups working on NOD2 have identified polymorphisms that show associations to CD. These variants seem to display altered structures of the LRR region, thus altering its response to bacterial LPS and leading to an inappropriate activation of the NF-κB pathway. Truncation variants lacking the LRR region have been shown to have a five-fold greater activation of NF-κB than the wild type NOD2 [47]. The identification of NOD2 as a susceptibility gene for CD provides the first clear link between the immune response to enteric bacteria and the development of the disease. Moreover, the hypothetical mechanics seem to agree with the current pathophysiological understanding of CD. However, the NOD2 frameshift mutation is quite rare, with only about 6.5% of CD patients carrying a homozygous mutation. It is estimated that the NOD2 mutation contributes to about one-fifth of the genetic risk for CD in the general population [46]. This means that other IBD genes must exist, and they remain to be uncovered.

1.2.2 SMOKING HABITS AND CD

Among the environmental risk factors that contribute to CD, smoking is one of the best documented. Somerville and colleagues, observed that patients with CD were more often smokers some 17 years ago [49]. Unlike its protective effects on UC, smoking has been documented as having a detrimental effect on CD. Several subsequent studies have demonstrated that there is a greater risk for CD among current smokers than in non-smokers [50][51]. Female smokers are at an even higher risk (3x) of developing CD compared to male smokers [52]. This difference has been tentatively attributed to oral contraceptive use, although this data is still inconclusive. Oral contraceptive use seems to be a risk factor by itself, and any possible interaction between smoking and oral contraceptive use requires further study [53]. It is also noteworthy that the duration of smoking and the number of cigarettes smoked can affect the site of CD. Small bowel and ileocolonic disease is more common among heavy smokers. As well, childhood or adult exposure to passive smoking appears to be an important determinant of

CD. Smoking behaviour is not only associated with the first outbreak of disease, but also influences the clinical course of CD. In one study, smokers with CD had a 34% higher relapse rate than non-smokers [54]. Smokers also have an increased risk for clinical, endoscopic, and surgical recurrences after surgical resection compared to non-smokers. Smokers were observed to develop more severe lesions at the site of anastomosis compared with non-smokers or ex-smokers [55]. In light of that, a study on smoking cessation in CD patients has determined that quitters were at lower risks of flare-ups than persistent smokers. As well, therapeutic needs and disease severity was lower in quitters and non-smokers, and they both had a better disease course than those who continued to smoke [56]. This proves that smokers with CD are not irreversibly affected, and that giving up the smoking habit can improve their clinical course.

Regarding pathogenesis, there are many routes by which smoking could influence the CD process, although possible mechanisms remain speculative. These include the immune response, the inflammatory cascade, intestinal motility, and others. Smoking has been found to influence cellular and humoral immunity, with alterations in immunoregulatory T-cells observed in heavy smokers. These changes could imply immune suppression, although this has not been studied in the setting of IBD. On the other hand, smoking and nicotine both reduce the production of pro-inflammatory cytokines. Smokers with active IBD have been documented as having significantly lower concentrations of IL-1β and IL-8. It is also well known that smokers have a greater capacity for free radical generation and reduced levels of antioxidants. As well, both smoking and nicotine have been shown to affect motility at various sites of the gastrointestinal tract. Finally, effects of smoking on the microvasculature of the bowel wall have been described in CD, and are consistent with ischemia [57]. However, it is still unclear how these changes, together or individually, affect the onset and course of CD on a physiological level. Much research is needed to elucidate the mechanisms of smoking in CD, and to find out whether nicotine is the main active ingredient responsible for the detrimental effects observed among smokers.

1.2.3 DIETARY FACTORS AND CD

Diet has been a major suspect among the putative environmental factors that could contribute to CD. Indeed, in genetically homogeneous populations, a change in dietary habits is one of the factors that might explain the increase in CD incidence over the past decades. Moreover, food is a major element affecting the intestinal environment, and the administration of certain types of special formula diets to CD patients often improves their condition. Over the years, several pre-illness dietary factors have been linked to CD risk, including refined sugar consumption, animal protein and fat intake, decreased fruit and vegetable consumption, among others. However, several of these theories were refuted, with only a few studies providing credible links to CD.

Certain studies provided evidence that high refined sugar consumption is a factor contributing to the development of CD. A high intake of simple sugars, both before and after diagnosis, is a remarkably consistent finding in patients with CD. Increased refined sugar consumption in CD patients was first reported by Martini and Brandes in 1976, and has since been backed by other studies [58]. Thornton and colleagues found that CD patients had a median intake of refined sugar that was almost double that of their matched controls (122gr/day versus 65gr/day) [59]. Katschinski and colleagues found a strong association between the consumption of added sugar and CD in non-smokers. A dose-response pattern was found, with the relative risk for CD increasing as the amount of added sugar increased [60]. However, there is no self-evident mechanism for the action of sugar to promote the development of CD, and the link between sugar and CD remains to be clarified. It is however noteworthy that most studies evaluating the effects of sugar intake also detected a decreased fresh fruit, vegetable and fiber consumption in CD patients. This, in turn, gave way to the supposition that a low consumption of dietary fiber may favor the development of CD. Nevertheless, a low refined sugar and high fiber diet was not found to influence the course of CD.

Another potential dietary factor is the 'western type' of diet, which is typically high in fat and protein. For instance, the introduction of western type

foods, or fast foods, may have contributed to the increasing incidence of CD in Japan since the mid-1960's. A recent study in Japan found that the increased incidence of CD was strongly correlated with increased average daily intake of total fat, animal fat, and n-6 polyunsaturated fatty acids. They also found a positive correlation with increased intake of animal protein, milk protein, but not fish protein, and a negative correlation was observed with the consumption of vegetable protein [61]. These results are supported by a subsequent study that, in addition, found a positive correlation between cholesterol intake and CD [62]. In racially homogeneous populations such as the Japanese, dietary changes can indeed be primary suspects in the increased incidence of CD.

When evaluating pre-illness dietary factors, one should keep in mind that in CD the lag between the onset of symptoms and diagnosis of the disease can sometimes exceed 5 years. So the dietary factors being scrutinized can be the outcome of modifications that patients gradually brought to their diets in the months or years before their symptoms became troublesome. It is therefore not surprising that the attempts to link specific dietary factors with the development of CD have run into major difficulties. Nonetheless, the findings of several studies suggest that dietary factors are important in the development of CD. Whether these factors have a primary effect in predisposed individuals or may simply modulate the effects of other environmental factors is yet unclear.

1.2.4 MICROBIAL INFECTIONS AND CD

Some of the most likely candidates for exogenous etiological factors in CD are microbial infections. It has been postulated that childhood infections can lead to IBD either by altering gut immuno-regulatory functions, or by leading to exposures to antibiotics and non-steroid anti-inflammatory drugs, which in turn may precipitate the disease. A case-control study by Wurzelmann and colleagues demonstrated an increased frequency of childhood infections and of antibiotic treatment among CD patients [63]. This is in concordance with the results of Ekbom and colleagues, who have found that patients with CD were more likely to have suffered infectious or non-infectious perinatal health

events [64]. There have been several attempts to uncover an infectious agent, whether viral or bacterial, which could trigger IBD in susceptible individuals. Candidate microbial pathogens included influenza A and B, herpes simplex virus, adenovirus, cytomegalovirus, *chlamydia psittaci*, *mycoplasma pneumonia*, *listeria*, *mycobacterium paratuberculosis*, mumps, rubella, measles and varicella viruses [63][65][66][67]. However, these experiments were unable to detect a significant link between the infectious agents and the development of CD. Despite these negative findings, *mycobacterium paratuberculosis* and measles virus remain the most debated potential pathogens in CD.

Mycobacterium paratuberculosis is the agent that causes Johne's disease in ruminants, which is quite similar to CD in humans. Several groups have detected *mycobacterium paratuberculosis* DNA by polymerase chain reaction in intestinal samples from CD patients, with rates varying between 0 and 72% [68]. Moreover, a potential transmission mechanism has been identified by recovery of *mycobacterium paratuberculosis* in 7% of commercially distributed pasteurized milk samples. However, the lack of convincing clinical, histochemical and immunologic evidence weakens the mycobacterium paratuberculosis theory. In addition, factors that should promote such a transmissible agent, including poor hygiene and sanitation, seem paradoxically to protect against CD.

Another infectious agent that was investigated was the measles virus, whose role as a causative agent has been much debated. A series of studies by Wakefield and colleagues suggested that there is a close link between measles virus and CD, on the basis of epidemiological and immunohistochemical findings [69][70][71]. According to this group, persistent measles viral infection causes focal granulomatous vasculitis that could lead to CD. It was believed that exposure to measles virus early in life could provide an opportunity for viral persistence. Furthermore, CD incidence seemed to increase in people born shortly after measles epidemics [70]. Some researchers even went so far as considering the measles vaccine as a possible etiologic agent, as several countries reported an increase in CD incidence after the introduction of live attenuated measles vaccination. However, the measles theory was soon enough discarded because the measles virus genome could not be detected in patients with CD [72].

Therefore, the present evidence for measles infection as an etiologic factor in CD is considered unconvincing. The same research group recently put forward the possibility of a possible relationship between paramyxovirus and IBD. In a cohort study, they determined that measles and mumps infections within the same year of life at a young age were significantly associated with later development of CD [66]. However, this theory remains to be proven, or refuted, by more sophisticated analyses.

Finally, there are numerous lines of evidence implying a key role of the enteric microflora in CD. It is believed that alterations of the bacterial milieu in the lumen could induce a chronic intestinal inflammation.

In a chronic inflammatory bowel disorder such as CD, it makes sense to suspect microbial agents in the pathogenesis. However, no single infectious agent has been solidly proven to trigger CD or render an individual more susceptible, and research in this direction still has much to uncover.

1.3 TREATMENT OF CD

At the present time there is no known cure for CD. Numerous treatment options are currently available that are directed towards controlling symptoms, addressing complications, and preventing relapses. Treatment of CD can be roughly broken down into five categories: pharmacological, biological, nutritional, surgical, and psychological. In this chapter, we will concentrate on the pharmacological, biological and nutritional treatments, as they are the most relevant forms of therapy for this study.

1.3.1 PHARMACOLOGICAL TREATMENT FOR CD

Given that CD is a multifactorial disorder with complex and incompletely understood pathogenesis, several classes of pharmacological drugs are employed in its treatment. These include aminosalicylates, corticosteroids, antibiotics, immunomodulators, and novel biologic agents. Pharmacological treatment for CD consists mainly of inductive therapies, which aim at inducing remission, and maintenance therapies, directed at maintaining remission in order to prevent relapses. It can also include treatment for perianal disease and prevention of post-operative recurrence. Generally, inductive therapies are administered according to disease severity and location.

Aminosalicylates are 5-aminosalicylic acid derivatives of sulfasalazine, and are considered primary therapies for the induction and maintenance of remission in mild to moderate CD. The precise in-vivo mechanisms of the aminosalicylates remain unclear. They have diverse anti-inflammatory properties such as inhibition of multiple pathways of arachidonic acid metabolism, suppression of IL-1 release, inhibition of leukocyte recruitment, and protection against oxidant injury. In vitro, sulfasalazine inhibits NF-κB [73].

Corticosteroids are the current mainstay of inductive therapy for moderate to severe CD. They suppress inflammation by several mechanisms through which they decrease eicosanoid production, inhibit the release of pro-

inflammatory cytokines, and decrease NF-κB production. Corticosteroids are superior to aminosalicylates in the treatment of moderate to severe CD. However, they are not effective at maintaining remission. Moreover, they produce several side effects that are mostly related to the dose and duration of the therapy [74]. However a new class of corticosteroids that has been developed is highly potent and rapidly metabolized, which limits its systemic bioavailability, thus resulting in fewer side effects. The one that has been most evaluated in clinical trials is budesonide, and it confers fewer side effects than conventional steroids, such as prednisone or prednisolone. However, it is less efficacious. Furthermore, as with conventional steroids, budesonide has been shown to be ineffective for the maintenance of remission or for the prevention of post-operative relapse in CD patients [75].

Since the enteric flora is thought of as a key promoter of the intestinal inflammatory response, it is logical that antibiotic therapy would be studied in CD. Metronidazidole is one of the most studied antibiotic agents for the treatment of CD, and it is believed to possess anti-inflammatory as well as immuno-suppressive effects. Metronidazidole therapy seems to be as effective as sulfasalazine and is most often beneficial to patients with perianal CD and fistulas. Ciprofloxacin is another antibiotic that has been mostly used in patients who are intolerant or unresponsive to metronidazole. It has been shown to be as effective at inducing remission as mesalazine in patients with mild to moderate CD, and has also been effectively used in combination with metronidazole [76].

Among the immunomodulatory agents, azathioprine (AZA) and 6-mercaptopurine are thiopurine analogues that have become increasingly important in the management of both active and quiescent CD, as well as for the maintenance of remission and the prevention of post-operative recurrence. The immunomodulatory effects of AZA and 6-MP lie in their metabolites, which are inhibitors of purine biosynthesis, and may be related to direct cytotoxicity and inhibition of cytokine synthesis [77]. Both drugs were also found to be effective steroid-sparing agents in patients with CD. Methotrexate is another drug with immunomodulatory and anti-inflammatory effects. It is an

inhibitor of dihydrofolate reductase and other folate-dependant enzymes, and has anti-inflammatory properties, which include decreasing IL-1 production, neutrophil chemotaxis, and eicosanoid production. Methotrexate seems to be beneficial in the short-term treatment of steroid-dependant or resistant patients and in maintenance therapy for CD. Cyclosporin A is yet another immunosuppressant, and it inhibits IL-2, IL-3, IL-4, interferon-γ and TNF-α. Since it has a rapid onset of action, it is a very attractive option for steroid refractory patients with severe UC.

1.3.2 BIOLOGICAL THERAPY FOR CD

Among the most recent theraputic advances are biological agents that target specific inflammatory factors. Anti-TNF therapies are of particular interest since this potent pro-inflammatory cytokine is believed to play a pivotal role in the initiation and perpetuation of intestinal inflammation in IBD. Infliximab is a high-affinity, chimeric (75% human, 25% murine) anti- TNF-α monoclonal IgG1 antibody. It acts by binding and neutralizing soluble TNF-α, causing the lysis of TNF-α-expressing cells, inducing T-cell apoptosis. Clinical trials have shown that a single 5mg/Kg injection of Infliximab induced clinical response in 81% of patients with moderate to severe CD [78]. Infliximab administered every 8 weeks increases long-term remissions as well. Patients receiving concurrent treatment with 6-mercaptopurine or azathiopurine may have a delayed recurrence, thus prolonging the response after Infliximab administration [79]. Infliximab has potential side effects, including upper-respiratory tract infections, abdominal pain, nausea, myalgias and fatigue. Up to 5% of patients may develop allergic reactions to infusions. Although there's no firm data linking Infliximab to malignancy, long-term follow up is necessary to establish Infliximab's efficiency and to identify all of its potential adverse effects.

Other biological therapies are under investigation, including CDP571, and agents that target various interleukins, NF-κB, and ICAM. CDP571 is a high-affinity humanized monoclonal IgG4 anti-body targeting TNF-α that has shown benefits in patients with active CD. The fact that it contains 95% human

and 5% murine sequences makes it theoretically less immunogenic than Infliximab. It has anti-TNF-α neutralizing activity but does not mediate antibody-dependant cytotoxicity [80]. Recently, large randomized trials demonstrated that CDP571 had potential benefits in some patients with refractory CD fistulas and in steroid-dependant patients.

Biological therapies targeting several anti- and pro-inflammatory interleukins are also currently under trial. IL-10 is an anti-inflammatory cytokine that suppresses cytokine production by Th1 cells, activated phagocytes, and non-immune cells [81]. However, its efficacy in clinical pilot trials has been disappointing. IL-11, another anti-inflammatory cytokine with thrombocytopoietic activity is also under investigation for CD treatment [82]. Another cytokine of interest is IL-12, which is pro-inflammatory and promotes interferon (IFN)-γ production and Th1 development. Anti-IL-12 therapy is currently under investigation. Trials with anti-NF-κB therapy, aimed at blocking this key transcription factor that affects a variety of genes encoding cytokines, chemokines, leukocyte adhesion molecules and inflammatory enzymes, are underway as well. Finally, trials with antisense oligonucleotides targeted against adhesion molecules are being carried out. ICAM-1 plays a central role in leukocyte trafficking and activation in the inflamed mucosa of patients with CD. ISIS-2302 is an engineered anti-sense oligonucleotide administered intravenously, which inhibits mRNA translation of ICAM-1. Although promising early results from trials in the treatment of CD were observed, larger randomized trials have been disappointing [83].

1.3.3 NUTRITIONAL THERAPY FOR CD

Nutritional considerations are intimately associated with CD since diet has been regarded as both a causative factor and a therapeutic agent. Nutritional treatment was initially employed in the management of CD because patients were often undernourished and suffered from growth retardation. Nutrition can be used either as primary or as adjunctive therapy for CD. In the context of primary therapy, it has mostly been used to induce remission in cases where

steroids were prohibited (osteoporosis, growth failure, diabetes) or for steroid-resistant CD. Adjunctive or supportive nutritional therapy, on the other hand, is indicated to improve the nutritional status or meet the nutritional requirements of patients, to stimulate growth, to reduce perioperative protein loss and morbidity, and to manage fistulae or post-surgical complications. Various dietary treatments can be administered with different compositions. Those most often employed include total parenteral nutrition (TPN), and defined formula diets which may be either elemental, semi-elemental or polymeric.

The hypothesis that intraluminal gut antigens or allergens contribute to the pathogenesis of IBD has led to nutritional interventions with a reduced antigenic load. TPN was viewed as a potentially effective therapy for achieving remission in patients with active CD because it could provide bowel rest while delivering adequate energy and essential nutrient requirements. Given that dietary and antigenic stimuli are decreased, the intestinal mucosa could potentially heal [84]. TPN was used as primary therapy to treat complicated cases of CD, and some studies observed high remission rates. However, TPN is also characterized by high relapse rates upon its discontinuation [23]. The use of TPN can lead to complications, as shown by experiments in animal models where it resulted in atrophy of the small intestinal mucosa [84]. Moreover, a prospective multi-center trial determined that bowel rest is not an essential contributing factor to remission, and the recognition of the importance of luminal nutrients for bowel integrity has weakened TPN's status as a primary therapeutic option [85]. However, TPN can be very efficient when used as an adjunctive therapy. When used in addition to steroids, it was found to prevent protein loss and preserve skeletal and respiratory muscle function in patients with active CD. When administered pre-surgically, TPN was also found to reduce perioperative complications in severely malnourished patients [86]. Patients that have undergone massive resections and short bowel syndrome are at risk for severe vitamin and electrolyte deficiencies. One of the major indications for long term home TPN is thus short bowel syndrome [87]. Finally, TPN can be administered in cases of post-operative fistulae which require bowel rest and nutritional support.

Elemental, semi-elemental, and polymeric diets can be administered either by tube or orally. These defined formulas were used in IBD to compensate for TPN's nutritional shortcomings. Elemental diets were initially administered to improve the nutritional status of CD patients awaiting surgery. When an improvement in disease activity was observed in addition to the nutritional benefits, elemental diets were considered to have a possible role as primary therapy. In elemental preparations, nitrogen is provided in the form of free amino acids, not requiring digestion. Moreover, this category of formulas generally contains low amounts of lipid, which is usually found in the form of medium chain triglycerides, requiring minimal luminal lypolysis and micellar solubilization. As with TPN, elemental diets confer no antigenic exposure and provides the patient with 'bowel rest' since it requires little enzymatic hydrolysis. However, in contrast to TPN, it results in fewer complications, is easier and less expensive to administer, and prevents intestinal mucosal atrophy. Children receiving an elemental diet display a greater linear growth, while adults receiving it present a positive nitrogen balance when compared to those on steroids [23]. The main downside to elemental formulas is their unpalatable taste, which most often leads to poor acceptance or compliance.

Semi-elemental diets differ from elemental formulae in that the amino acids have been replaced by short peptides of varying amino acid mixtures. Since elemental diets have a higher osmotic load than semi-elemental diets, they could exacerbate the patients' diarrhea. By virtue of their lower osmolality and a more efficient nitrogen source, semi-elemental formulas have been theorized to be nutritionally more effective than elemental diets [88]. Polymeric diets, on the other hand, are comprised of whole proteins, normal lipid content, fiber, and such. There is no 'bowel rest' in the case of polymeric diets, although the antigen load is much reduced compared to a normal diet.

Exposure of the gut to dietary substrates has beneficial trophic effects [84]. For instance, glutamine, which is an essential nutrient for intestinal epithelial cells, has a positive effect on intestinal structural integrity and immune function. SCFA are the energy substrates of choice for colonocytes and can promote their growth and repair [89]. Other important nutrients found in semi-elemental and

polymeric formulas include dietary fibre, epidermal growth factors, and nucleotides, all of which exert a beneficial effect on the gastrointestinal system [86].

All three types of defined formulas have been used to treat growth retardation and to induce remission in CD. The ensuing increase in lean body mass may actually be important in order to reduce disease activity, since immune response in children suffering from malnutrition is closely linked to their protein status. The various forms of liquid diets have been compared to each other and the results have been conflicting as to which is the better treatment option. Giaffer and colleagues reported that the administration of an elemental diet to patients with active CD achieved a 75% remission rate, while a polymeric diet conferred benefit in only 36% [90]. Raouf and colleagues, on the other hand, found that both elemental and polymeric treatments led to similar remission rates [91]. Other studies even suggested that polymeric were superior to elemental diets. Further large controlled trials are needed to compare elemental, semi-elemental, and polymeric diets in terms of their efficacy and to shed light on the mechanisms of their action.

Comparisons of these formulas as primary therapy for CD versus steroid therapy have provided conflicting results. Whereas defined formula diets have generally displayed convincing results in the induction of remission, the same cannot be confirmed when it comes to maintaining long-term remissions. Despite potential side effects, drug therapies still induce the highest rates of remission and the quickest relief of symptoms. Therefore, medications remain the primary treatment of choice for the majority of patients with active CD. Several trials have demonstrated that TPN, elemental, semi-elemental, and polymeric diets can provide many benefits when used in conjunction with drug therapy.

Chapter 2

Immune Dysfunction in Crohn's Disease

2.1 The Mucosal Immune System Under Physiological Conditions

To better understand the processes involved in mucosal inflammation, we shall begin by describing mucosal immune function under physiological conditions. We shall explore the morphology of the intestinal mucosa, the cells that comprise the mucosal immune system and their distribution within the mucosa. Then we will discuss the key cytokines and their respective roles in mucosal immunity. Finally, we shall describe the immune processes and imbalances implicated in the pathogenesis of CD, including the role of NF-κB.

2.1.1 Morphology of the Gastrointestinal Tract

The gut luminal surface is very convoluted, greatly increasing the surface area available for absorption. Starting at the stomach, a single layer of epithelial cells covers the luminal surface. This epithelial layer constitutes the first line of defence against foreign antigens and microbial pathogens. Just below the epithelium is the lamina propria, layers of connective tissue through which pass blood vessels, nerve fibers, and lymphatic ducts. The lamina propria is separated from the underlying tissues by a thin layer of smooth muscle cells, the muscularis mucosa. These three layers, the epithelium, lamina propria, and muscularis mucosa, are usually referred to collectively as the mucosa. Beneath this is the sub-mucosa, which is a second layer of connective tissue containing a network of nerve cells (submucosal plexus), blood and lymphatic vessels. Next is a smooth muscle layer, the muscularis externa, which provides the forces for moving and mixing the gastrointestinal contents. It is formed by two layers, the circular and longitudinal muscles, separated by a network of nerve cells known as the myenteric plexus. Finally, a thin layer of cells and connective tissue surrounds the outer surface of the GI tract, called the serosa.

With respect to Crohn's disease, the main area of focus is the mucosa, where most of the immune processes occur. Villi, finger-like projections of the

surface of intestine, are covered with a single layer of epithelial cells that are linked at the luminal border by tight junctions. Epithelial cells are themselves covered by small projections referred to as microvilli on their brush border. Each intestinal villus is drained by a single lymphatic vessel called the lacteal, and by a capillary network. The epithelial cells lining the gut are continuously being renewed. Mature cells at the tips of the villi are replaced by cells that arise from the base of the crypts. These cells differentiate into mature, absorptive enterocytes as they migrate towards the villus tip, replacing senescent cells that undergo apoptosis. In this manner, the entire epithelium of the small intestine is replaced every 5 days. In all, the combination of folded mucosa, villi, and microvilli increases the small intestine's absorptive surface area by about 600 fold as compared to a flat-surfaced tube of the same dimensions. This translates into a small intestinal surface area of about 300 square meters [92].

2.1.2 CELLS OF THE MUCOSAL IMMUNE SYSTEM

The intestinal mucosa must serve as a barrier between the internal and external environments and constitutes an important first line of defence against infections. Until recently, the epithelial cell was considered a passive partner whose functions are affected by products of immune cells. However, it is now known that epithelial cells produce cytokines such as TGF-β and IL-6, which have profound effects on lymphocytes, and that they have receptors for several cytokines on their surfaces [93][94]. Epithelial cells can express MHC class II receptors, and their role in antigen presentation has been proposed [95]. Intestinal epithelial cells are capable of responding to a wide array of biologically active agents found in the lumen, including bacteria and their products, cytokines, and short-chain fatty acids. Therefore, epithelial cells should be included in the discussion of mucosal immunity despite the fact that they are not immune cells.

The intestinal epithelium is continuously exposed to an abundant, complex bacterial flora, which varies quantitatively and qualitatively along the length of the intestine [96]. It represents the major stimulus to the development of the intestinal immune system. Consistent with its huge antigenic load, the

intestine is rich in lymphoid cells, which constitute 25% of the total cells present. Indeed, the immune system of the gastrointestinal tract represents the largest mass of lymphoid tissue in the body. Collectively, this is referred to as the gut associated lymphoid tissue (GALT). GALT comprises various cell types, which are mainly categorized into phagocytes and lymphocytes [97]. Phagocytes include neutrophils, monocytes and macrophages, all of which form part of the innate immune system and provide non-specific immunity. Macrophages are strategically placed where they will encounter their targets. They phagocytose and degrade antibody-coated and complement-opsonized foreign particles, and kill microbes by secreting a host of enzymes, reactive oxygen radicals and lipid derived mediators. However, the oxygen radicals they secrete are potentially detrimental if not controlled, causing lipid peroxidation and membrane destabilization. This results in increased membrane permeability in the inflamed intestine. Macrophages can also behave as antigen presenting cells, and secrete cytokines that promote activation of T cells. Lymphocytes are comprised of B, T and natural killer (NK) cells, which mediate what is called adaptive or specific immunity. B cells differentiate in the bone marrow and are then transported to peripheral lymphoid organs by the circulation. Upon activation, B cells secrete antibodies, which are then disseminated all over the body to guide the immune response (via phagocytes, NK cells or complement) that eliminates the foreign antigens. There are two sub-populations of B cells: the memory B cells, and the activated plasma cells. As for the T cells, they differentiate in the thymus into different sub-populations, the T helper ($CD4^+$) cells and the cytotoxic/suppressor (CD8+) cells, the ratio of which is normally 2:1. T cells lack receptors for antigens and require the presence of antigen-presenting cells. Cytotoxic T cells will bind the antigen-presenting cells and are capable of killing them directly. Suppressor T cells inhibit the function of both B cells and cytotoxic T lymphocytes via the secretion of cytokines. As for helper T cells, they help activate both B cells and cytotoxic T cells. Helper T cells have three subtypes: Th1, Th2 and Th0 with distinct functions distinguished by their cytokine profiles. IFN-γ and IL-2 are the cytokines characteristic of Th1 cells, while IL-4 and IL-5 are typical of Th2 cells. Th1 and Th2 cells also reciprocally

regulate one another via cytokines such as IFN-γ and IL-10, and the balance between these two subsets may be very important in maintaining mucosal homeostasis. Crohn's disease is believed to be a Th1-mediated disease, given that IFN-γ and IFN-γ producing cell numbers are both increased [97]. A third population of lymphocytes, NK cells, have important functions in host defence against viruses and tumors. These cells arise in the bone marrow and do not manifest specificity for antigen; they attack and kill target cells directly. Their participation in an immune response is enhanced by cytokines secreted by activated helper T cells.

2.1.3 Anatomic Distribution of Immune Cells in the Mucosa

Lymphocytes can be found in three main regions: scattered between epithelial cells, dispersed throughout the lamina propria, and in organized collections in the lamina propria, termed Peyer's patches [99]. Cells at each site have distinct phenotypic and functional characteristics. The cells found in the epithelial layer, also known as intraepithelial lymphocytes (IEL), represent about one of every six to ten cells in the epithelium. The majority of IEL are T cells, while plasma cells are absent and B lymphocytes infrequent. IELs are located in a site that would render them exposed to a variety of antigenic stimuli, providing the first line of immune interaction. The inflammatory cells populating the lamina propria are comprised of T cells, B cells, plasma cells, as well as macrophages, dendritic cells, eosinophils and mast cells. There is strong evidence that these T cells regulate the mucosal immune response to luminal antigens and have migrated to the mucosa from the mesenteric lymph nodes. One interesting fact is that the lamina propria is the only place where large numbers of plasma cells are present continuously. They differentiate from B cells in the lamina propria, under the effect of cytokines derived from T helper cells and epithelial cells.

Peyer's patches are lymphoid follicles which very much resemble those found in the spleen or lymph nodes. They contain all of the cells needed for immune induction, including B cells, T cells, macrophages and dendritic cells.

The B cells are usually located in the center of the patch, while T cells predominate in the interfollicular areas. These B lymphocytes represent about 60-70% of cells in Peyer's patches, with T lymphocytes representing about 20%. Unlike other follicles, Peyer's patches lack afferent lymphatics. Instead, they have a specialized follicle-associated epithelium (FAE) which pinocytoses luminal antigens and delivers them to the Peyer's patches. These FAE are characterized by the lack of goblet cells and the presence of M cells, which are flattened, fenestrated cells without microvilli. They appear to be induced from enterocytes by close interactions with B lymphocytes. M cells do not function as antigen presenting cells; they simply transfer the antigenic material to lymphocytes in the Peyer's patches [100]. The presence of M cells therefore renders bacterial organisms more accessible to Peyer's patches, which serve as sites for the induction of mucosal immune responses.

An important feature of the GALT is mucosal lymphocyte 'homing' after activation. Lymphocytes that have been induced in the Peyer's patches will travel to the mesenteric lymph nodes where they will undergo further division and maturation. However, they will also selectively 'home' back to the intestine and other mucosal sites [101]. The mechanism of selective lymphocyte homing into mucosal tissues is dependent upon the expression of certain cell adhesion molecules on lymphocytes that will interact with related ligands on endothelial cells. Inflammation increases the expression of certain such receptors, such as ICAM-1 and ELAM-1, facilitating leukocyte entry into areas of intestinal inflammation .

2.2 Cytokines and Nuclear Factor Kappa-B

The following section examines a few of the molecular components of the GALT system, namely cytokines and nuclear factor kappa B (NF-κB).

2.2.1 Cytokine Classification

Antigen presentation is closely followed by the generation of cytokines, which provide signals enabling communication among the different cells involved in the immune response. Cytokines are pleiotropic proteins, between 5 and 50 kDa, with autocrine, paracrine and endocrine activities. They regulate immune and inflammatory responses, but also other processes such as cellular proliferation, differentiation and apoptosis. Cytokine secretion is usually a brief and self-limited event, influencing the synthesis and actions of other cytokines. Their actions can sometimes be redundant, such that several cytokines can share the same role during inflammation. External signals regulate the expression of cytokine receptors and cell responsiveness to cytokines. Responses to cytokines generally consist of changes in gene expression in target cells, which in turn induce their expression of new functions and/or proliferation [102]. Macrophages are a major source of cytokines, but epithelial cells and T helper cells also produce a number of these molecules.

Cytokines are generally categorized according to their main function, mostly into pro-inflammatory and anti-inflammatory (or immunoregulatory) subgroups, chemokine groups, or as Th1 vs. Th2 cytokines. Pro-inflammatory cytokines include IL-1, IL-6, IL-8, monocyte chemotactic peptide-1 (MCP-1) and TNF-α, while IL-4, IL-10 and transforming growth factor-beta (TGF-β) are considered anti-inflammatory cytokines. However, some pro-inflammatory cytokines can also have a role in controlling inflammation, such as IL-1, which has a role in tissue healing, and IL-6, which can induce the secretion of anti-inflammatory acute phase proteins [103].

Chemokines, or chemotactic cytokines, are small (8-12 kDa) pro-inflammatory proteins that can stimulate and guide the migration of leukocytes to sites of inflammation. They can be categorized into 3 groups based on having one or two cysteine residues in their amino terminal domain, and whether or not the latter are adjacent (CC) or separated by an intervening amino acid between the first two cysteines (CXC). Members the CC family, such as MCP-1, MCP-3, and macrophage inflammatory protein 1 (MIP-1) are regulated upon activation. Normal T cells express and secrete RANTES, which functions mainly as a chemoattractant for monocytes, eosinophils, subsets of T lymphocytes and basophils. The CXC family members, including IL-8, GRO-α, GRO-γ, and epithelial cell-derived neutrophil activator 78 (ENA-78), are mainly important for the chemoattraction and activation of neutrophils. There also is the C chemokine lymphotactin, which serves primarily to attract T lymphocytes [104].

Th1 and Th2 lymphocytes develop from precursors, native CD4+ T lymphocytes (Th0). Their pattern of differentiation depends on the stimuli present early during an immune response. Th1 cells secrete IFN-γ, IL-1 and TNF-α, and mediate delayed hypersensitivity, while Th2 cells produce IL-4, 5, 9, 10 and 13, which influence B cell responses. Th0 lymphocytes secrete both of the above classes of cytokines [102].

2.2.2 FUNCTIONS OF CYTOKINES IN THE IMMUNE SYSTEM

TNF-α is one of the principal cytokines mediating the acute inflammatory response to gram-negative bacteria and other microbes, and is responsible for many of the systemic complications of severe infections. It is produced mainly by activated mononuclear phagocytes, and stimulates the recruitment of neutrophils and monocytes to sites of infection where it will activate these cells to eradicate microbes. The most potent stimulus for TNF production by macrophages is lipopolysaccharide (LPS), an endotoxin found at the surface of gram-negative bacteria. TNF stimulates endothelial cells and macrophages to secrete a host of chemokines, as well as IL-1 by mononuclear

phagocytes. In addition to its role in inflammation, TNF induces apoptosis in some cell types [102].

Interleukin-1 is similar to TNF-α considering that it is mainly produced by mononuclear phagocytes, and that its principal function is to mediate host inflammatory responses to infections and other inflammatory stimuli. Unlike TNF however, IL-1 is produced by many other cells including neutrophils, epithelial and endothelial cells. There are two forms of IL-1, IL-1α and IL-1β, that are less than 30% homologous to each other but mediate the same biologic activities. The effects of IL-1 are the same as those of TNF, but depend on the quantity of IL-1 secreted. In low concentrations, it functions as a mediator of local inflammation, whereas when secreted in larger quantities, it enters the blood stream and exerts endocrine effects. Monocytes also produce a natural inhibitor, the IL-1 receptor antagonist (IL-1ra), that functions as a competitive inhibitor for IL-1 [102].

Interferon-γ is the principal macrophage-activating cytokine, acting mainly as an effector cytokine. It is produced primarily by NK cells, CD4+ Th1 cells, and Cd8+ T cells. IFN-γ provides the means by which T lymphocytes and NK cells activate macrophages to kill phagocytosed microbes, stimulate expression of MHC-class I and class II on antigen presenting cells, promote the differentiation of Th0 into Th1 cells, and inhibit the proliferation of the Th2 cells. In all, it promotes macrophage-rich inflammatory responses [102].

Other pro-inflammatory cytokines including IL-6, 8, 12, 15, 18, and GRO-α, contribute to the inflammatory process mainly by stimulating the activation and proliferation of various immune cells, and by directing them towards sites of inflammation.

Among anti-inflammatory cytokines, TGF-β is produced by activated T cells, macrophages and intestinal epithelial cells. Its main functions are the inhibition of the activation and proliferation of activated T and B cells, intestinal epithelial cells and macrophages. It also inhibits adhesion molecule and MHC class II expression [93].

Interleukin-4 is the classical cytokine defining the Th2 subset. It is also produced by activated basophils and mast cells. IL-4 stimulates IgE production by B cells and Th2 cell proliferation. Its main anti-inflammatory role involves the suppression of macrophage by antagonizing IFN-γ, thereby inhibiting cell-mediated immune reactions [102].

Interleukin-10, on the other hand, is mainly an anti-inflammatory cytokine that is produced by macrophages, T lymphocytes, and some non-lymphoid cells. It provides negative feedback to activated macrophages by inhibiting their IL-12 and TNF production, and by preventing the expression of MHC class II molecules on their surface. IL-10's main function is the maintenance of homeostatic control of innate and cell-mediated immune responses [102].

Other anti-inflammatory cytokines include IL-13, which is structurally similar to IL-4. It is produced by Th2 cells and some epithelial cells, and mimics the effects of IL-4 on macrophages by inhibiting their activation and antagonizing IFN-γ. The balance between pro- and anti-inflammatory cytokines is of crucial importance for the maintenance of immune homeostasis. Under physiological conditions, the secretion of anti-inflammatory cytokines follows that of pro-inflammatory cytokines and mediates down-regulation of inflammation, wound healing and tissue repair.

Table 1 Selective characteristics of cytokines that play a role in mucosal immunity and intestinal inflammation

Cytokine	Main cellular source	Main target cell	Dominant Functions
IL-2	T cells	T cells, all IL2R bearing cells	T cell activation, proliferation, clonal expansion, and differentiation
IL-4	T cells, mast cells, basophils	Multiple cell types	TH2 differentiation, mediation of allergy, and immunosuppression
IL-7	Stromal cells	T cells	T cell proliferation and cytotoxicity
IL-15	Most cells	IL-2R bearing cells	Multiple activities
IL-1	Monocytes, macrophages	Most cells	Mediation of infectious and inflammatory processes
IL-6	Multiple cells	Most cells	Enhancement of immunoglobulin production, and immunoregulation
TNF-α	Macrophages	Multiple cells	Mediations of inflammatory and cytotoxic responses
Chemokines	Immune, nonimmune cells	Leukocytes	Selective chemoattraction
IFN-γ	T cells, natural killer cells	Most cells	Induction of MHC class II antigens, monocyte activation, TH1 differentiation and IL-4 suppression
IL-12	Phagocytes, B cells	T cells	TH1 differentiation, mediation of infectious responses
IL-18	Multiple cells	Multiple cells	IL-12-like effect
IL-10	Monocytes, macrophages	Multiple cells	Anti-inflammatory activity and immunosuppression
CSF	Stromal cells	Granulocytes and macrophages	Growth, differentiation, and survival of hematopoietic cells
IL-5	T cells, mast cells	Eosinophils, mast cells, basophils	Mediation of allergic and parasitic diseases
IL-9	TH2 cells	T cells, mast cells	Undefined
IL-11	Hematopoietic stromal cells	Multiple cells	Stimulation of intestinal crypt cells
IL-13	T cells	Multiple cells (except T cells)	IL-4-like effect
IL-16	T cells	$CD4^+$ cells	T cell chemokinesis
IL-17	T cells	Unclear	Pro-inflammatory

Excerpt from Podolsky DK, and Fiocchi C. [105]

2.2.3 NUCLEAR FACTOR KAPPA B

NF-κB comprises a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory responses. NF-κB exists in the form of heterodimers and homodimers of Rel proteins, of which there are five family members in mammalian cells: RelA (p65), cRel, RelB, NF-κB1 (p50) and NF-κB2 (p52). Studies in knockout mice have shown distinct functions for different members of the NF-κB/Rel family. The predominant form of NF-κB is the p65/p50 dimer, the most potent gene transactivator among the family. This heterodimer is also the major NF-κB prototype in the nucleus of cytokine-stimulated intestinal epithelial cells [106]. Under resting conditions, NF-κB is held in an inactive state, sequestered in the cytoplasm by a family of regulatory proteins known as inhibitors of kappa B (IκB). There are seven IκB variants (IκBα, IκBβ, IκBε, IκBγ, Bcl3 p105 and p100) that preferentially associate with various Rel family dimers [107]. Different IκB molecules have distinct and overlapping specificities. The most abundant NF-κB inhibitors are IκBα (37 kDa), IκBβ and IκBγ (45 kDa each). However, the most characterized and most potent NF-κB inhibitor is IκBα, which binds avidly to the p65 subunit of NF-κB. During an immune response, several stimuli, including TNF-α, IL-1 and LPS, activate a cascade of events that will end with the translocation of NF-κB into the nucleus where it can perform its transcription functions. For starters, stimulation of cells will activate a complex of IκB kinases (IKK) that phosphorylate inducible IκBα on the amino terminus at serine residues 32 and 36. Phosphorylation of these serine residues is the first step in IκB degradation. Phosphorylated IκB is then ubiquinated and rapidly degraded through the cell's 26S proteasome complex. This proteolytic degradation of IκB allows the release of NF-κB which will transmigrate into the nucleus [108]. Once in the nucleus, NF-κB will transactivate target genes for several cytokines and factors, as well as genes for its own inhibitor, IκBα. This

will allow for a negative feedback loop with restoration of IκBα levels and binding of nucleic NF-κB, thereby down-regulating NF-κB activation [109].

2.2.4 ROLE OF NF-κB IN INFLAMMATORY DISEASES

NF-κB is activated by a wide range of agents, including IL-1, TNF-α, LPS, double-stranded RNA, phorbol esters, cyclic AMP, UV light, oxidants, bacteria and viral transactivators. Once it is activated, NF-κB transcriptionally regulates cellular genes implicated in early immune, acute phase and inflammatory responses. Among the genes that are transcribed by NF-κB are included those for IL-1, TNF-α, IL-2, 6, 8, and 12, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), T cell receptor-α (TCR-α), and MHC class II molecules [110]. As well, cytokines that are stimulated by NF-κB, such as IL-1 and TNF-α, can also directly activate the NF-κB pathway, thereby providing a positive feedback loop that can amplify the inflammatory response and increase the duration of chronic inflammation. Moreover, NF-κB stimulates the production of enzymes such as iNOS and COX-2, which generate nitric oxide and prostanoids respectively, contributing further to the pathogenesis of the inflammatory process [111].

Table 2 Inducers of NF-κB

Cytokines and Growth Factors	Bacteria and Bacterial Products
Interleukin-1β *	*Salmonella* and *Shigella* *
Interleukin-2 *	Enteropathogenic *E. coli* *
Interleukin-17 *	Helicobacter pylori
Interleukin-18 *	Mycoplasma fermentans
Lymphotoxin	Listeria monocytogenes *
Leukotriene B4 *	Lactobacilli
Tumor necrosis factor-α *	Lipopolysaccharide *
Macrophage colony stimulating factor	Peptidoglycan-polysaccharide *
Platelet-derived growth factor *	Toxic shock syndrome toxin 1
T Cell Mitogens	
Antigen	Viruses and Viral Products
Anti-CD2	Adenovirus *
Anti-CD3	Epstein-Barr virus
Anti-CD28	Human immunodeficiency virus type 1
Calcium ionophores	Human T cell leukemia virus type 1
Lectins	Hepatitis B virus B
Oxidative stress	Herpes simplex virus type 1
Hydrogen peroxide *	Double-stranded RNA
Ozone	Latent membrane protein
Reactive oxygen intermediates *	

* Documented to stimulate intestinal epithelial cells.
Excerpt from Jobin C, and Sartor RB. [112]

Table 3 Molecules regulated by NF-κB in IEC

Cytokines and chemokines
Interleukin-1β
Interleukin-6
Interleukin-8
GRO-α, GRO-β
RANTES
Macrophage inflammatory protein-2
Cell surface receptors
Interleukin-2R
CD95/APO-1 (Fas)
Adhesion molecule
ICAM-1
Inflammatory enzymes
Inducible nitric oxide synthase
Cyclooxygenase-2
Stress proteins
Complement factors B, C3, C4
Immunoregulatory molecule
Major histocompatibility complex I and II

Excerpt from Jobin C, and Sartor RB. [112]

Given its propensity to exacerbate inflammatory responses, it is no surprise that the activation of NF-κB is involved in the pathogenesis of several chronic inflammatory diseases, including rheumatoid arthritis, asthma and IBD. The presence of several pro-inflammatory cytokines in the synovial fluid and lungs of patients with these disorders supports a role for the NF-κB pathway [113]. NF-κB activation has also been documented in mucosal biopsy specimens of patients with IBD [114]. Moreover, altered NF-κB regulation may be involved in the pathogenesis of disorders such as atherosclerosis and Alzheimer's disease, where the host's inflammatory response is at least partially involved in the pathogenesis. Indeed, NF-κB may play an important role in the initiation and progression of the fibroproliferative process in atherosclerosis and neuritic plaques and neuronal apoptosis in Alzheimer's disease, through its effects on genes coding for inflammation and cell proliferation [115][116].

In addition to its role in gene activation in the context of an immune or inflammatory response, NF-κB is a key mediator of genes involved in the control of cellular proliferation and apoptosis. Indeed, NF-κB is involved in protecting cells from undergoing apoptosis in response to cytokine treatment or DNA damage. For instance, following treatment with TNF-α, NF-κB activation can increase the expression of anti-apoptotic cellular proteins, thereby reducing apoptosis [117]. However, other groups have attributed pro-apoptotic properties to NF-κB. Inhibition of NF-κB by certain antioxidants prevented apoptosis in human thymocytes and promyelocytic leukemia cells [118]. This dual role for NF-κB in apoptosis can be explained by the possibility that different NF-κB members mediate different signals. As well, the role of NF-κB in apoptosis depends on the cell type or type of stimulation that determines which signalling pathways are activated.

NF-κB has also been proven to be involved in cell differentiation and growth. In certain cases, NF-κB was believed to be a promoter of cellular differentiation, whereas in others, it was thought to be an inhibitor. For instance, the inhibition of NF-κB activity in keratinocytes prevented the

maturation process of these cells, while another study confirmed that NF-κB functions as a negative regulator of skeletal muscle differentiation [119][120]. In fact, whether NF-κB promotes or represses cellular differentiation most probably depends on which specific forms of this factor are represented during the development of a particular tissue, and on cellular differences with regards to transcriptional response.

Whatever its effects on cellular differentiation and growth, NF-κB has also been associated with oncogenesis, since abnormalities in the regulation of the NF-κB pathway have been witnessed in a variety of human cancers. These abnormalities resulting in constitutively high levels of NF-κB in the nuclei of tumors have been demonstrated in breast, ovarian, prostate, and colon cancers [121]. Indeed, aberrant levels of NF-κB/Rel have been reported in primary breast cancers [122]. As well, NF-κB has been shown to be a downstream target of the oncogenic Ras, and to be required for Ras-mediated cell transformation [123]. Similarly, the chimeric oncoprotein Bcr-Abl, implicated in acute lymphoblastic and chronic myelogenous leukemias, also requires NF-κB to induce cellular transformation [124]. This suggests that a key role for NF-κB in the malignant transformation of cells is highly plausible; however, it is yet unclear whether over-activation or excessive expression of this transcription factors in transformed cells is directly linked to the transformation, or whether NF-κB only provides an accessory signal.

In summary, NF-κB plays a key role in controlling the expression of multiple inflammatory and immune genes involved in asthma, rheumatoid arthritis, atherosclerosis, cancer, AIDS, toxic shock, and acute phase responses. It is therefore a favourable target for therapeutic intervention, and intense research is being carried out to develop NF-κB member-specific and cell type-specific drugs that can prevent the deleterious effects NF-κB on its target cells.

2.3 IMMUNOPATHOGENESIS OF CROHN'S DISEASE

The precise nature of the initiating events involved in CD remains unknown, although findings in animal models and human studies support the concept of immunodysregulation. It is believed that the aberrant immune response may be driven by exposure to the bacterial flora or to dietary antigens. What is established, however, is that increases in epithelial permeability, pro-inflammatory cytokine levels and NF-κB activity are present in CD. In this section, we discuss these alterations and their impact on the disease.

2.3.1 MUCOSAL BARRIER DISRUPTION IN CD

The epithelial layer that lines the surface of the intestinal mucosa constitutes the first line of defence against the numerous antigens and microbes found in the intestinal lumen. The stability of this epithelial barrier is established by the formation of tight intercellular junctions, zona adherens and desmosomes. The integrity of the epithelium and its tight seal against luminal contents are functional prerequisites of the healthy bowel. On the other hand, most studies have provided evidence for increased intestinal permeability in patients with active CD [125][126]. Gassler and colleagues found that the expression of the zona adherens and tight junction proteins were downregulated in epithelial tissue of CD patients [127].

In addition to the paracellular route (through tight junctions), uptake via endocytosis is thought to be a dominant mode of transport through the epithelium. Under physiological conditions, limited and controlled uptake of protein fragments is essential for the surveillance of antigens in the gastrointestinal tract. This sampling is mostly mediated by M cells, but epithelial cells also have the capacity to present antigen to the underlying immune cells. Therefore, a disturbance in permeability could reflect increased uptake of antigens via enterocytes or tight junctions. Soderholm and colleagues found an increased uptake of ovalbumin in the non-inflamed mucosa of CD patients.

This occurred via both paracellular and transcellular routes [128]. Other groups have confirmed alterations in intestinal permeability in areas lacking macroscopic injury [129][130]. These reports and the description of abnormal permeability among healthy first-degree relatives of CD patients have led to the hypothesis that aberrant permeability might represent a primary defect in CD [131][132]. However, others established that increased intestinal uptake is secondary to the inflammation in CD [133]. Moreover, increased cytokine release by activated immunocytes could induce the aberrant permeability of the mucosal barrier. Cytokines such as IFN-γ, IL-4, and TNF-α adversely affect barrier function in cultured human epithelial cells [134][135][136]. These cytokines are known to be increased in CD; as such their implication to epithelial barrier dysfunction in IBD is very plausible. Whether an epithelial barrier defect is a primary disturbance or a secondary phenomenon in CD remains unclear. The implication is that such defects provide an opportunity for luminal antigen to cross the epithelial barrier, causing an excessive inflammatory response.

2.3.2 CYTOKINE LEVELS IN CD

The state of physiological inflammation in the gut mucosa depends on the balanced secretion of Th1 and Th2 cytokines. The chronic pathological inflammation in IBD is theoretically associated with an imbalance in Th1/Th2 cytokines. Several studies have reported that CD is mainly associated with a Th1 response. Some studies have shown that levels of IL-2 protein and mRNA are consistently higher in CD, compared to UC patients, and LPMC from CD patients exhibit an exaggerated reactivity to IL-2 stimulation [137][138]. Elevated IFN-γ mRNA, spontaneous IFN-γ protein secretion, and high levels of IFN-γ-producing cells in tissues from CD patients have been reported [139][140][141]. As well, LPMC from CD patients have been shown to produce low levels of IL-4, and T cells from CD patients seem to be resistant to IL-4 inhibitory activity [142][143]. All of these changes in cytokine profile point to the existence of Th1 hyper-reactivity in CD. Th1 cells are partially responsible for the release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α from responding

macrophages, and contribute to the amplification of pre-existing mucosal inflammation. However, the existence of a Th1 pattern is not definitive, since early post-surgical ileal lesions from CD patients were shown to have an opposite Th2 response, with high IL-4 and low IFN-γ levels [144]. Therefore, there is a lack of convincing proof of classifying CD as a purely Th1 disease.

Table 4 Cytokine levels in patients with Crohn's disease

Cytokine	Direction of change	References
IL-1	↑	[150]
IL-2	↑	[137][138]
IL-4	↓↑	[142][143][144]
IL-6	↑	[152]
IL-8	↑	[145]
IL-10	↑	[153]
TNF-α	↑	[150][151]
IFN-γ	↓↑	[139][140][141][144]
TGF-β	↑	[154]
ENA-78	↑	[147]
MCP-1	↑	[148]
MIP-1	↑	[149]
RANTES	↑	[149]
IL-12	↓	[149]

Aside from the alterations observed in Th1 cytokines, changes in several chemokines have been observed in IBD. Chemokines are key molecules responsible for the recruitment and influx of immune cell to sites of inflammation, and their involvement in IBD represents a major component of its pathogenesis. Most of the attention has been focused on IL-8, the levels of which are markedly increased in CD mucosal samples, correlating with the degree of inflammation [145]. Macrophages are the major source of IL-8 in CD, but intestinal epithelial cells also contribute [146]. Moreover, intestinal epithelial cells produce substantial levels of another chemokine, ENA-78, in both CD and UC [147]. Since ENA-78 is a neutrophil attractant, this indicates a direct involvement of epithelial cells in the pathogenesis of CD. Several other chemokines are increased in CD, including MCP-1, RANTES, and MIP-1 [148][149]. In all, chemokines are clearly involved in CD, but their role is non-specific, likely secondary to initiating events.

Pro-inflammatory cytokines are considered of great importance to the pathogenesis of CD. Increased levels of IL-1β and TNF-α have been documented in the mucosa of CD patients [150]. As well, elevated TNF-α levels were detected in sera of some diseased patients [151]. IL-6 is another pro-inflammatory cytokine that is increased in tissues and sera of CD patients [152]. Cytokines such as these contribute to IBD by activating both immune and non-immune cells, and by up-regulating the expression of cellular adhesion molecules. As well, TNF-α may be especially important in promoting tissue injury through cellular apoptosis. TNF-α-induced injury of the epithelium perpetuates inflammation through impaired barrier function, leading to uptake of pro-inflammatory substances from the lumen. Quite like chemokines, pro-inflammatory cytokines are believed to be a secondary event since the greatest increases are detected after the inflammation is histologically detectable.

Normally, the secretion of anti-inflammatory cytokines ensues following the acute inflammatory response, counteracting the effects of pro-inflammatory cytokines and reinstituting homeostasis in the affected tissues. Among anti-inflammatory mediators are Th2 cytokines such as IL-4, IL-10, and IL-13, which oppose Th1 functions and inhibit the production of IFN-γ. While IL-4 levels are generally decreased, IL-10 levels seem to be increased in both tissues and sera from CD patients [153]. Increased mucosal content of TGF-β1, another anti-inflammatory mediator, has also been found in active [154]. IBD is characterized by an imbalance of IL-1 and its antagonist, in that IL-1ra levels are not sufficiently raised to compensate for the increased amounts of IL-1β [155]. This imbalance between pro- and anti-inflammatory cytokines translates into a failure of down-regulation and reparative processes, resulting in ongoing inflammation that typifies CD.

2.3.3 Increased NF-κB Activation in CD

Several lines of evidence suggest that NF-κB activation of cytokine genes is an important contributor to the pathogenesis of CD. Several studies have

documented increased activation of NF-κB in the intestine of CD patients and in rodents with experimental colitis. One study by Rogler and colleagues reported a significantly greater activation of the p65 subunit in mucosa from CD patients as compared to normal mucosa; this activated form of NF-κB was localized to macrophages and intestinal epithelial cells [156]. This presence of activated NF-κB in epithelial cells supports results from in-vitro studies where activation of NF-κB in intestinal epithelial cells could be induced by IL-1β and TNF-α [157]. Moreover, the experiments by Rogler and colleagues showed that activated NF-κB is concentrated in intestinal epithelial cells located in the crypts, and that it correlates with the degree of mucosal inflammation. Another group reported ex-vivo expression of NF-κB in isolated bowel endothelial cells from CD patients [158]. Thus, NF-κB activation can be seen at many levels of the intestinal mucosa.

One of the ways to assess NF-κB activity in disease is by evaluating its transcription end products. NF-κB is clearly one of the most important regulators of pro-inflammatory gene expression, and its involvement in the synthesis of pro-inflammatory cytokines has been widely documented. Indeed, NF-κB regulation of genes encoding TNF-α, IL-1β, IL-6, IL-8, MCP-1, VCAM-1, ICAM-1, macrophage colony-stimulating factor, and macrophage-granulocyte colony-stimulating factor has been evidenced by numerous studies [159][160][161][162][163]. Several of these cytokines are increased in CD, which implies a function for NF-κB in up-regulating inflammation in this setting. NF-κB mediated increases in IL-8 and ICAM-1 expression and secretion following infection of the human intestinal epithelial cell lines HT-29, Caco-2, and T84 with enteroinvasive bacteria [164]. The inhibition of NF-κB activation with an IκBα super-repressor blocked the expression of IL-8, GRO-α, MCP-1, ICAM-1, COX-2, NOS2, and TNF-α in response to bacterial infection. This involvement of NF-κB in up-regulating inflammation was the basis for trials aimed at inhibiting the pathway. *In vitro* treatment of lamina propria macrophages from IBD patients with p65 anti-sense oligonucleotides reduced

pro-inflammatory cytokine production [165]. As well, NF-κB-directed therapy has been effective in a murine model of IBD induced by 2,4,6-trinitrobenzene sulfonic acid, where local application of p65 anti-sense oligonucleotides blocked both clinical and histological evidence of disease activity [158]. In light of these results, the anti-inflammatory effect of inhibition of NF-κB confirms that activation of NF-κB, and of cells expressing it, is fundamental to inflammatory processes. Indeed, NF-κB provides a common signal transduction pathway for the up-regulated expression of pro-inflammatory genes and mediators in CD. However, it is unclear whether the increase in NF-κB activation observed in CD is in response to a causal agent or to increased immunological signalling.

In conclusion, the pathogenesis of CD is complex and consists of three interacting elements: genetic susceptibility, priming by luminal antigens, and immune-mediated tissue injury. In such a setting, intestinal inflammation arises from abnormal immune reactivity to bacterial flora or dietary antigens in the intestine of individuals who are genetically susceptible. In healthy individuals, the intestinal mucosa is in a state of controlled inflammation with maintenance of mucosal homeostasis in the face of a host of such luminal antigens. However, in the genetically susceptible individual, there seems to be an aberrant response to normal luminal contents, leading to extensive inflammation and tissue injury. CD patients exhibit disrupted mucosal barrier functions, altered cytokine profiles, and increased NF-κB activation. Several theories have suggested a role for enteric bacteria in the initiation of disease by over-activation of immune cells in susceptible individuals. However, other hypotheses propose a role for oxidative stress in the disruption of the epithelial layer and mucosal defences, thereby unleashing a series of events that will culminate in chronic inflammation. It is this involvement of oxidative stress in the pathogenesis of CD that will be discussed in the next chapter.

Chapter 3

Oxidative Stress and Crohn's Disease

3.1 Description of Oxidative Stress

Radicals have been implicated in the toxicities related to numerous chemicals and in the pathogenesis of many diseases. The list of disorders in which radicals and oxidants have been implicated is still growing, in part because these reactive molecules can account for most of the tissue damage that has been identified in a variety of diseases [166]. Oxidative stress has been widely implicated in CD, especially in light of the imbalance in oxidant/antioxidant status and increased lipid peroxidation that have been documented in patients. This chapter is dedicated to the understanding of oxidative stress in Crohn's disease. We shall begin with a definition of oxidative stress, follow with an explanation of the effects of oxidative stress on NF-κB, and conclude with a documentation of the evidence for oxidative stress in CD and its treatment with antioxidants.

3.1.1 Sources of oxidants and oxidative damage in biological systems

In the human body, normal biological activities as well as exogenous factors lead to the abundant formation of radicals every day. Radicals are potentially dangerous since they can lead to oxidation and subsequent damage to DNA, proteins, lipids and other major biological constituents. This injury has been implicated in various conditions such as ageing, cardiovascular disease, cataracts, cancer, immune system decline, and brain dysfunction [167].

A radical is a chemical species with an unpaired electron that can be neutral, positively or negatively charged. There are several types of radicals, including oxygen-, thiyl-, carbon-, nitrogen-, and phosphorus-centered radicals. These radicals sometimes have a role as oxidants; however not all radicals are oxidants, and not all oxidants are radicals. The prevalence of oxygen in biological systems results in oxygen-centered radicals being the most common type. Reactive oxygen species (ROS) and reactive oxygen metabolites (ROM) are

both terms used by scientists to include not only the oxygen-centered radicals, but also some non-radical derivatives of O_2. The most common ROM found in biological systems include the hydroxyl radical ($HO\cdot$), the superoxide anion (O_2^{-}), and hydrogen peroxide (H_2O_2), all of which are electron reduction products of singlet oxygen (O_2). Other radicals and oxidizing species commonly found include lipid alkoxyl ($LO\cdot$) and peroxyl ($LOO\cdot$) radicals, lipid carbon centered radical ($L\cdot$), semiquinone radical ($HQ\cdot$), and nitric oxide (NO) [168]. All of these species have different chemical reactivities and lifetimes, which affect the type and extent of damage they can produce in biological systems. Radical lifetimes can vary from extremely short to indefinitely long. For instance, the hydroxyl radical is extremely reactive, with a half-life of 10^{-9} seconds. This makes the hydroxyl radical quite unselective in the damage it causes. On the other hand, the superoxide anion exists 10 000 times longer than the hydroxyl radical, and semiquinone radicals, which are found in cigarette tar, can have almost infinite lifetimes.

Thus, radicals do not usually react with great selectivity, which makes them all the more destructive. They have a potential for self-sustaining chain reactions, consisting of initiation and propagation reactions. Initiation reactions result in the creation of species with unpaired electrons, while propagation reactions result in the maintenance or transfer of the radical character. Propagation reactions account for the majority of free radical formation. However, this process can be stopped by a termination reaction, which will result in the conversion of radicals into spin-paired products.

There are various sources of oxidants in biological systems, and these can be divided into endogenous and exogenous sources. The endogenous sources that account for most of the oxidants produced by cells are represented by phagocytic cells, mitochondrial electron transport, peroxisomes, microsomal electron transport, soluble oxidase enzymes, and cytochrome P-450 enzymes. However, under physiological conditions, the first two systems represent the largest source of radical production *in vivo*, while the others contribute mostly under disrupted conditions. Phagocytic cells are perhaps the best recognized biological sources of free radicals. In the presence of bacteria- or virus-infected

cells, activated phagocytes exhibit a marked increase in oxygen consumption. This 'oxidative burst' results in the production of nitric oxide, superoxide anion, hydrogen peroxide, and hypochlorous acid (HOCl), and is meant to destroy the invading microbes [169]. However, the radicals liberated from phagocytes can cause damage beyond their target cells, injuring surrounding tissues, perpetuating inflammation. Therefore, excessive phagocytic activity can have deleterious effects on the affected host. It has also been established for quite some time that the mitochondrial electron transport system is capable of generating ROS [170]. Superoxide anion, hydrogen peroxide, and hydroxyl radical are the inevitable by-products of normal aerobic respiration by mitochondria. It is believed that up to 2% of total mitochondrial oxygen consumption goes toward the production of ROS [171]. However, some scientists believe that this figure was calculated under a highly artificial system, and revised it to a much lower percentage of ROS produced under physiological conditions [167]. Studies on the leakiness of electron transport chains were based on experiments with bacteria and mitochondria respiring in air-saturated solutions, and might represent overestimations [172]. Nonetheless, the fact remains that normal mitochondrial activity contributes to the oxidative load of an organism.

Among the exogenous sources that may significantly contribute to increases in oxidant load are transition metals, nitrogen oxides, and natural phenolic compounds. Redox-active metal ions such as iron and copper are essential enzyme co-factors. Under normal conditions, these metals are trapped under the action of chelating factors, so that no free metals are found in body fluids [171]. When free in biological systems, these iron and copper salts have noxious capacities, given that they facilitate the generation of oxidizing radicals from peroxides through Fenton-type reactions. An example of such a process would be iron-dependant Fenton chemistry, whereby superoxide dismutates to form hydrogen peroxide, which is subsequently reduced by ferrous iron to form a hydroxyl radical (Figure 1). This process is known as the iron-catalyzed Haber-Weiss reaction [173].

$$2\,O_2^{\cdot-} + 2\,H^+ \rightarrow H_2O_2 + O_2$$
$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + OH^- + Fe^{2+}$$

Figure 1 – Iron-catalyzed Haber-Weiss reaction

Given its propensity for oxidative reactions, iron can be very destructive in humans, and iron overload has been associated with several pathologies, including cirrhosis, impaired cardiac function, diabetes mellitus, and endocrinopathies. Iron-catalyzed free radical-mediated lipid peroxidation seems to play a significant role in these diseases [174][175]. Another exogenous source of pro-oxidants is nitric oxide from cigarette smoke, which can cause oxidation of macromolecules and deplete antioxidant stores. Natural phenolic compounds found in plants, such as chlorogenic and caffeic acid, can also generate oxidants through redox cycling [176]. There are thus several sources that promote the oxidant load in humans, although the importance of each of these radicals in any specific disorder remains unclear.

As mentioned above, radicals and oxidants are capable of reacting with proteins, DNA, and lipids, accounting for a vast array of changes that result in cellular and tissue damage [166]. The oxidation of proteins can result in loss of sulfhydryl groups and modifications of amino groups, which in turn lead to the formation of carbonyls and other oxidized moieties. Oxidized proteins are normally removed by proteolysis, although the proteolytic enzymes that hydrolyze them are not sufficient to prevent age-related accumulation of oxidized proteins [177]. When the rate of protein oxidation exceeds their removal, cell injury becomes apparent, as is reflected in diseases associated with premature aging such as Werner's syndrome and progeria. This increase in oxidized protein levels may also reflect unrepaired damage to other cellular components, including DNA [178].

Oxidative damage to DNA is inevitable, given that antioxidant defences in the human body are not always optimal. This damage includes single- and double-strand breaks, base changes, deletions, and chromosomal aberrations [179]. In fact, free radicals are estimated to modify approximately 10,000 DNA base pairs every day [180]. The major molecular mechanisms involved are direct

reaction of hydroxyl radicals and carbonyl compounds with DNA. Superoxide and hydrogen peroxide only react with DNA in the presence of transition metals which allow hydroxyl radical formation. DNA repair enzymes, known as glycosylases, normally excise the abnormalities. However, their removal is not entirely efficient and some oxidized DNA will accumulate with age. Eventually, this leads to cell death or the potential development of malignancies [181].

Lipid peroxidation is one of the best established examples of a radical chain reaction that plays a role in biological changes. The term "peroxidation" was attributed to lipid autoxidation, because its main products are generally lipid peroxides. The introduction of radicals into a system containing polyunsaturated fatty acids (PUFA) will result in a chain reaction represented by the autoxidation of many PUFA molecules (Figure 2).

Initiation

$$\text{Initiation} \rightarrow 2R^{\cdot}$$
$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$$
$$ROO^{\cdot} + LH \rightarrow ROOH + L^{\cdot}$$

Propagation

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$
$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$$

Termination

$$LOO^{\cdot} + AOH \rightarrow LOOH + AO^{\cdot}$$
$$AO^{\cdot} + LOO^{\cdot} \rightarrow \text{Non - Radical Products (NRP)}$$

Figure 2 – Chemical equations involved in lipid peroxidation

During lipid peroxidation, a radical (LOO·) will react with a PUFA (LH) molecule by abstracting a doubly allylic hydrogen atom, thereby creating a lipid hydroperoxide and a carbon-centered radical. This process is part of the propagation step and will occur repeatedly until a termination reaction takes place. Hence, a single initiation event can result in the conversion of hundreds of fatty acid side chains into lipid hydroperoxides. The formation of lipid peroxides in biological membranes results in changes in membrane fluidity and permeability, as well as inactivation of membrane-bound receptors and enzymes [182]. Lipid peroxidation is also associated with increased rates of protein degradation and mutagenicity. Numerous studies have documented the

occurrence of lipid peroxidation *in vivo*, and this system has been implicated in the pathogenesis of numerous disorders, including atherosclerosis, cancer and Alzheimer's disease.

3.1.2 ANTIOXIDANT DEFENCE SYSTEMS

Living organisms have evolved several defence mechanisms to limit the levels of reactive oxidants and the damage they inflict. Antioxidants may act at different levels in the oxidative process by scavenging free radicals, binding metal ions, or removing oxidatively damaged biomolecules. Whereas some antioxidants are endogenously synthesized, others have to be provided by the diet. Antioxidant defence systems can be divided into enzymatic and non-enzymatic categories. Enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), and glutathione transferase (GST) while non-enzymatic antioxidants include the low-molecular weight radical scavenger glutathione (GSH), as well as dietary antioxidants such as ascorbic acid, α-tocopherol, carotenoids, ubiquinols, and flavonoids. In biological systems, these defence mechanisms act in concert to limit oxidative processes and to minimize the deleterious effects of oxidants.

Enzymatic antioxidants typically remove excess $O_2^{\cdot-}$ and H_2O_2 (Figure 3). Superoxide dismutase is an enzyme that exists under two different isoforms: Mn SOD contains manganese at its active site and is located in the mitochondria, while Cu-Zn SOD contains copper and zinc at its active site and is mostly present in the cytosol [172]. SOD enzymes remove $O_2^{\cdot-}$ by accelerating its conversion into the less potent H_2O_2, thereby protecting the organism from the toxic actions of $O_2^{\cdot-}$. Since SOD enzymes generate H_2O_2, they must act in collaboration with H_2O_2-removing enzymes such as catalase and GSH-Px. Catalase is a ubiquitous heme protein usually located in peroxisomes, which is responsible for the conversion of H_2O_2 to oxygen and water [183]. However, the most important H_2O_2-removing mechanism is the enzyme glutathione peroxidase (GSH-Px). It has a selenocysteine residue at its active site, which is essential for proper enzyme activity. Its main function consists of H_2O_2 removal

using reduced glutathione (GSH) as a substrate, with the end products being water and oxidized glutathione (GSSG). GSH-Px can also convert lipid hydroperoxides into alcohol and water via the same process. Reduced glutathione is subsequently regenerated by the glutathione reductase (GSSG-Red) enzyme, which uses NADPH as a source of reducing power. Excess GSSG can sometimes be transfered to the extracellular medium by GST in order to maintain the cells' redox status. GST is a multifunctionnal cytosolic, microsomal and membrane-bound detoxifying enzyme that can be considered a back-up enzyme to catalase and GSH-Px.

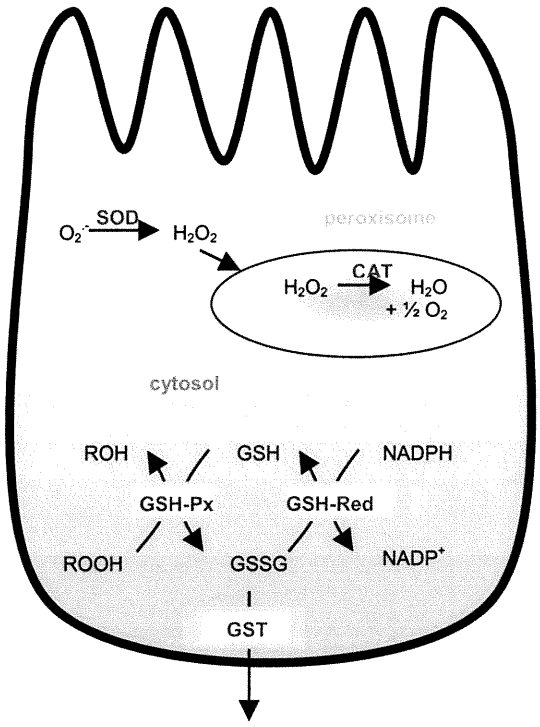

Figure 3 – Endogenous antioxidants within an enterocyte

In addition to antioxidant defence enzymes, mammalian cells are equipped with low-molecular mass antioxidants that can be of endogenous or exogenous origin. These can be divided into lipid- and water-soluble

antioxidants, depending on the compartments within which they perform their activities. Examples of water-soluble antioxidants include GSH and ascorbic acid, while vitamin E, carotenoids, ubiquinols, and flavonoids represent lipid-soluble antioxidants. The tripeptide GSH is the most important intracellular antioxidant, and can scavenge free radicals directly as well as serve as a substrate for the enzymes glutathione peroxidase and glutathione-S-transferase [184]. The ratio of GSH/GSSG in tissues is normally maintained high (100:1). However, severe oxidative stress may overcome the ability of the cell to reduce GSSG to GSH, leading to a depletion of GSH [185]. Ascorbic acid is present in most body fluids and plays essential metabolic roles *in vivo*. However, dietary sources are essential, as it is not synthesized in humans. Its biochemical importance is imparted by its strong reducing potential, which makes it an efficient free radical scavenger. In addition, it can protect lipids and membranes from oxidative damage by scavenging lipid peroxidation initiating radicals [184]. What's more, both ascorbic acid and glutathione participate in the regeneration of lipid-soluble antioxidants by reducing their respective radicals [186]. However, ascorbic acid can become a pro-oxidant in the presence of transition metal ions, acting as a reducing agent and generating $O_2^{\cdot-}$, H_2O_2, and $HO^{\cdot}$. But since transition metal ions are not generally available in the extracellular fluids of the human body, the antioxidant properties of ascorbate predominate [171].

Regarding lipid-soluble antioxidants, α-tocopherol is the most abundant form of vitamin E, and is an essential nutrient in humans. It is the most important free radical scavenger within membranes and lipoproteins [187]. Alpha-tocopherol is considered to be the major chain-breaking antioxidant of lipid peroxidation, with one molecule of α-tocopherol being able to scavenge two molecules of peroxyl radical. The α-tocopherol radical is less reactive than the lipid peroxyl radical and therefore slows the chain reaction. Carotenoids and retinoids protect the cells from oxidative stress by quenching singlet oxygen and scavenging peroxyl radicals [187]. Ubiquinols are also effective lipid-soluble chain-breaking antioxidants, with membrane concentrations sometimes comparable to those of vitamin E [184]. As for flavonoids, they are versatile antioxidants that

can prevent lipid peroxidation by scavenging initiating radicals, chelating metal ions, scavenging lipid peroxyl radicals, or inhibiting enzymatic systems responsible for free radical production [184].

In conclusion, cells can tolerate mild oxidative stress, which usually up-regulates the synthesis of antioxidant defence systems in an attempt to restore balance. However, severe oxidative stress can produce major derangements of cell metabolism, including DNA strand breakage, damage to membrane transporters, enzymes and other proteins, as well as peroxidation of lipids. As a result, cell mutagenesis or death may occur. In most human diseases, oxidative stress is a secondary phenomenon, as a consequence of disease activity. Nonetheless, it is also a significant contributor to cell injury and death. Therefore, the importance of oxidative stress must not be undermined and further studies into the exact mechanisms of oxidative damage and possible antioxidant treatments are essential for the understanding of numerous pathologies including CD.

3.2 OXIDATIVE STRESS AND NUCLEAR FACTOR KAPPA B

It has become increasingly apparent that oxidants can play an important role in mediating specific cell responses and expression of genes involved in degenerative pathologies such as inflammatory diseases and cancer. For quite some time now, the role of oxidants in mediating inflammatory reactions has been studied, and recognized, in a variety of experimental models. Several inflammatory stimuli have been examined – including cytokines, bacterial toxins, ischemia-reperfusion, and lipid mediators. Induction of oxidative stress was believed to be central to all inflammatory processes, regardless of the stimulus. However, one topic that has drawn a great interest in recent years concerns the effects of oxidative stress on the regulation of gene expression. In particular, the transcription factor NF-κB has been implicated in the transcriptional up-regulation of inflammatory genes in response to oxidants or changes in cellular redox status. Given the scarcity of published results on the interaction of NF-κB and oxidative stress in the setting of IBD, we shall begin with a generalized approach to the topic of oxidative activation of NF-κB, and subsequently discuss experimental data on the redox modulation of NF-κB activity and cytokine production in CD.

3.2.1 OXIDANT MODULATION OF NF-κB ACTIVATION

Numerous studies indicate that ROS may serve as common intracellular agents that contribute to the process of NF-κB activation in response to a diverse range of stimuli (Figure 4). The hypothesis regarding redox modulation was based on four lines of evidence [188], which taken together provide confirmation for the involvement of ROS in many physiological pathways related to NF-κB activation.

First, direct addition of H_2O_2, itself a source of ROS, results in activation of NF-κB in several cell lines. Indeed, H_2O_2 was shown to be an effective inducer of NF-κB in the Wurzburg subclone of Jurkat T cells, L6 skeletal

muscle myotubes, human breast MCF-7, HeLa cells, and 70Z/3 pre-B cells [189][190][191][192]. Considering that various cell types may respond to H_2O_2 in different ways, there may be a number of mechanisms by which it might activate NF-κB. Indeed, H_2O_2 was shown to induce TNF release from T lymphocytes, to cause and increase of intracellular [Ca^{2+}] in Jurkat subclones, and augment GSSG levels in several cell types, all of which could contribute to NF-κB activation [188]. In addition, H_2O_2 seems to induce tyrosine kinase and phosphatidylinositol 3-kinase activation, both of which might be important pathways in H_2O_2-mediated NF-κB activation. Although H_2O_2 can activate NF-κB, this effect is highly dependent on cell type, possibly with distinct mechanisms involved in different experimental models.

Second, in some cell types increases in ROS have been reported in response to agents that activate NF-κB. Both IL-1 and TNF have been shown to increase ROS in primary human fibroblasts, and IL-1 led to ROS production in lymphoid and monocytic cells [193][194][195]. As well, LPS led to an increase in H_2O_2 production in B-cell lines and H_2O_2 release has been demonstrated in response to NF-κB activating agents in other systems [196][197]. Thus, certain stimuli that activate NF-κB also induce ROS production, this response is still cell- and stimulus-specific phenomenon.

Third, inhibition of NF-κB activation by antioxidant compounds has been reported by several groups and constitutes one of the most compelling lines of evidence for a role for oxidative stress in NF-κB activation. Antioxidants such as pyrrolidine dithiocarbamate (PDTC), N-acetyl-L-cysteine (NAC), vitamin E derivatives, vitamin C, butylated hydroxyanisole (BHA), o-phenanthroline, and α-lipoic acid have been shown to inhibit NF-κB activation in various cell types [188][198]. Yet, no particular antioxidant has been shown to have universal effects, and NF-κB inhibition is still cell- and stimulus-dependent. In addition, compounds that inhibit NF-κB at specific points in a pathway also have multiple or unexpected targets on that pathway, so that

inhibitory effects cannot truly be attributed to the compound's antioxidant properties.

Finally, the inhibition or over-expression of endogenous antioxidant enzymes has been shown to modulate NF-κB activation by some agents. In certain cell lines, for example, over-expression of SOD inhibited TNF-induced NF-κB, while in others it potentiated the effect [191][197]. Others have shown that catalase over-expression inhibited TNF activation of NF-κB [197]. More recently, a role for glutathione peroxidase has been suggested in the attenuation of some pathways of NF-κB activation [199]. This role was particularly attributed to phospholipid hydroperoxide GSH-Px, indicating that lipid peroxides, rather than H_2O_2, may have a role in particular pathways to NF-κB [199]. This suggests that much of the evidence for the role of H_2O_2 and ROS in the activation of NF-κB could be attributed to lipid peroxidation.

Taken together, these points denote that certain, but certainly not all cell types, respond to oxidative stress by up-regulation of NF-κB activity. One suggested mechanism through which ROS may mediate NF-κB activation is via the modulation of protein kinases involved in the phosphorylation of the inhibitory IκB, although the exact pathways remain to be elucidated [200]. Information obtained so far indicates that critical steps in the signal transduction cascade are sensitive to oxidants and antioxidants, with these effects being specific for cell-type and stimulus. Thus, oxidative stress does not seem to be central to all NF-κB activation pathways as was previously postulated, but rather modulates certain pathways in particular environments. However, the molecular basis for redox regulation of NF-κB is largely unknown, and further experimentation is required to clarify the exact mechanisms of these processes.

3.2.2 NF-κB ACTIVATION BY ROS IN CD

Current evidence indicates that intracellularly generated ROS are implicated in the regulation of transcription factors involved in inflammatory responses. In the setting of pathologies such as CD, the redox modulation of NF-κB could induce the transcription of a variety of inflammatory factors that contribute to, and perpetuate, the state of chronic inflammation that is typical of the disease. Unfortunately, few studies have investigated oxidant activation of NF-κB in IBD. Only one group investigated redox modulation of NF-κB in human intestinal smooth muscle (ISM) cells. Natarajan and colleagues have shown that smooth muscle cells isolated from the ileum of patients with CD exhibited heightened levels of cellular activation characterized by spontaneous transcription of IL-6, and secretion of the pro-inflammatory chemokine IL-8 [201]. They also reported that Crohn's disease ISM (CDISM) cells exhibited spontaneous activation of NF-κB, with a 400% increase in IL-8 promoter activity as compared to normal human ISM (NHISM) cells. Because NF-κB is an oxidant-sensitive transcription factor, and their studies revealed that NF-κB was spontaneously activated, they hypothesized that CDISM cells were under chronic oxidant stress. To prove their point, they used the antioxidant compound PDTC to shift the redox equilibrium and examine its effects on NF-κB. They demonstratied that at low concentrations (25 μM to 100μM), PDTC suppressed NF-κB-driven IL-8 promoter activity in a dose-dependent fashion. In addition, at 50μM, PDTC inhibited the DNA-binding activity of NF-κB. Their findings therefore suggest that CDISM cells are under oxidative stress and that an antioxidant such as PDTC could alter the redox equilibrium to a more reduced state, thereby attenuating the spontaneous activation of NF-κB. This study clearly demonstrated that oxidant activation of NF-κB, albeit *in vitro*, does take place in CD and that it leads to the production of inflammatory factors. Whether this mechanism is persistent *in vivo* remains unclear. Further experimentation is warranted in order to clarify the role of oxidative stress in the pathophysiology of IBD.

In summary, ROS act as second messengers in the inflammatory process in CD. Factors that shift cellular redox equilibrium toward oxidized states activate NF-κB and a host of other oxidant-sensitive transcription factors, while agents that sway the balance toward more reduced intracellular environments inhibit NF-κB. This redox modulation of NF-κB results in the transcription of a variety of inflammatory factors, which inevitably contribute to the perpetuation of inflammatory responses.

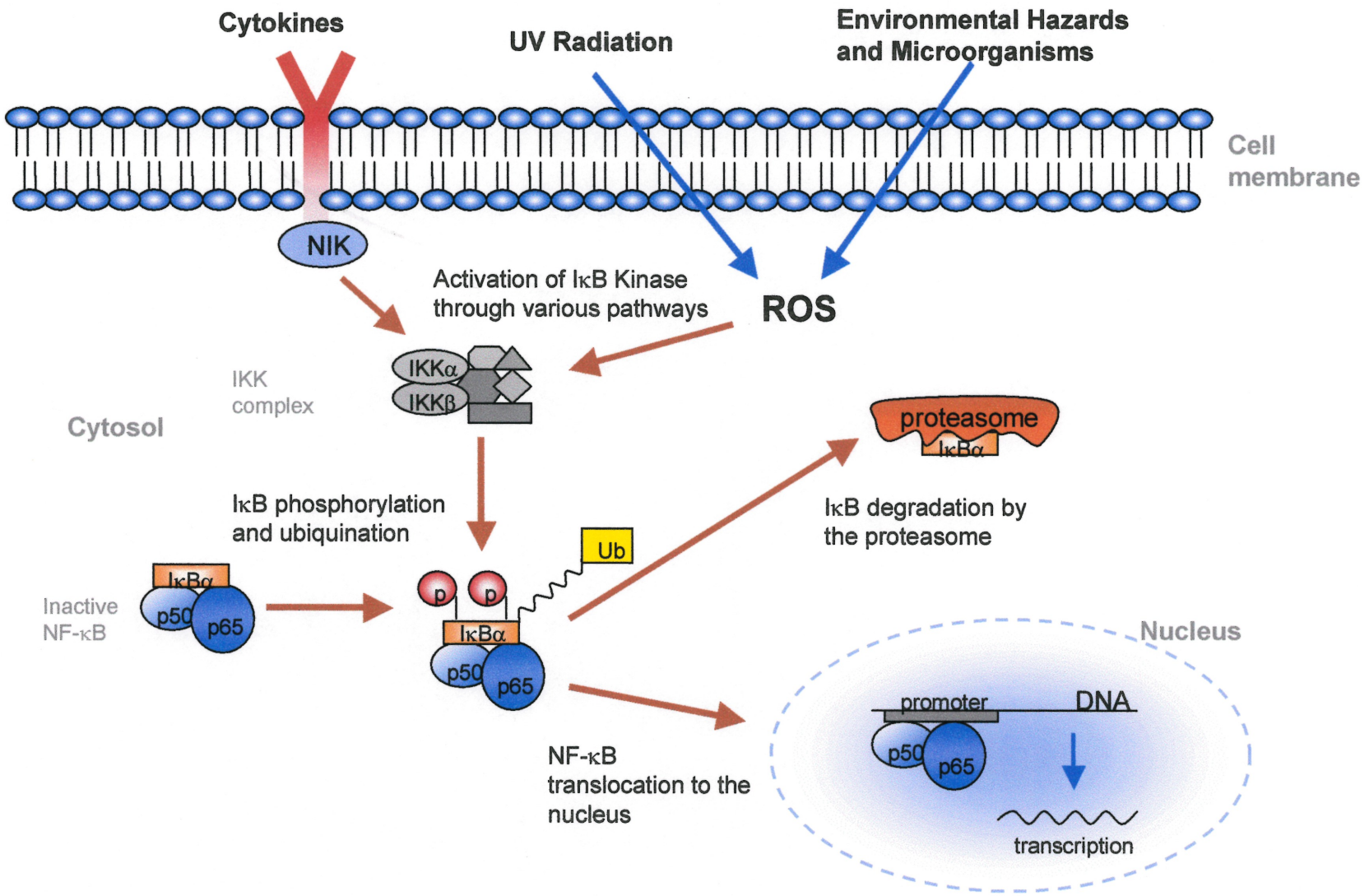

Figure 4 - Activation of NF-κB.

3.3 OXIDATIVE STRESS AND ANTIOXIDANT THERAPY IN CD

Several recent studies have emphasised the potential pathogenic role of free radicals in IBD, suggesting that ROM have a central role in the inflammatory response. By activating the transcription factor NF-κB, they initiate the expression of genes controlling several aspects of the inflammatory and acute phase responses. However, there has been a lack of conclusive evidence regarding the primary role for ROM in the pathogenesis of CD, and there still is considerable debate as to whether free radicals are involved in the pathogenesis of tissue injury, or are simply generated as a consequence of damaged tissues. Nevertheless, quite a few studies have established the presence of oxidative stress in CD as evidenced by increased oxidant production and decreased antioxidant status in patients. Moreover, antioxidant therapy and treatment with drugs having radical-scavenging properties seem to improve disease activity. These data imply an important role for oxidative stress in the pathogenesis of CD. This part of the chapter will be devoted to the discussion of pro-oxidant/antioxidant status in CD patients, and the effects of several antioxidant treatments.

3.3.1 INCREASED ROM PRODUCTION AND OXIDATIVE DAMAGE

There have been numerous studies on free radical production in CD, most of which have confirmed an overproduction of ROM in the inflamed intestine. Keshavarzian et al and Simmonds et al have demonstrated that the inflamed colons of human patients and of animals with experimental colitis produce much larger amounts of ROM than controls or uninvolved intestinal segments [202][203]. Their results suggest furthermore that neutrophils are predominantly responsible for the production of excessive amounts of ROMs in the inflamed colon. The data in the literature seems to be inconsistent with reference to ROM production by neutrophils, particularly peripheral blood neutrophils, with some groups reporting an increase and others a decrease in

production [203][204][205][206]. These discrepancies may be explained by differences in experimental procedures, but also suggest that events in peripheral blood do not reflect those in the inflamed intestinal mucosa. One group recently found that peripheral neutrophils from CD had a significantly higher oxidative response therein response to different stimuli [204]. Their results suggested that circulating neutrophils are primed in active disease as well as in remission. Other groups have demonstrated that circulating monocytes contribute to the oxidative load as well, possibly contributing to the formation of intestinal lesions in CD [207][208].

The presence of oxidative stress has also been supported by the lipid peroxidation, protein oxidation, and DNA damage in tissue. Wendland et al reported that breath pentane, ethane and F_2-isoprostane outputs were significantly higher in CD patients, representing an increased peroxidation of n-3 and n-6 PUFA [209]. Mingrone et al, on the other hand, reported a decrease in non-protein respiratory quotient, which reflects increased fat oxidation and might explain the reduced body fat stores in CD patients [210]. Other studies have measured the production of malondialdehyde (MDA), which is a by-product of lipid peroxidation. One group reported elevated MDA levels in rectal biopsies and another in blood samples from CD patients [211][212]. Regarding other substrates that are susceptible to oxidation, Lih-Brody et al found elevated levels of ROM and protein carbonyls in both inflamed and non-inflamed mucosa of CD patients as compared to controls [213]. They also observed an increase in DNA oxidation, as evidenced by elevated levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) in actively inflamed lesions. Their results are in concordance with those from other groups who reported increased protein thiol oxidation in mucosal cells and augmented 8-OHdG concentrations in leukocyte DNA from CD patients [214][215]. Most importantly, 8-OHdG is promutagenic and could have an important role in CD complications, considering the increased incidence of intestinal cancers in IBD [216]. Aside from DNA oxidation, researchers have found that the constitutive and oxidant-induced activity of adenosine diphosphate ribosyl transferase (ADPRT), an enzyme involved in DNA repair, is reduced in patients with IBD, which could

contribute further to the extent of damage caused by DNA oxidation [217]. Finally, the finding of increased ROM production capacity and protein carbonyl content in uninvolved mucosal sections suggests that these findings are not just the consequence of active inflammation, but might actually precede histologic inflammation. Indeed, the oxidation of constituent proteins may contribute to the pathogenesis of CD by chemoattraction and activation of immunocytes, as well as through the disruption of enzyme systems necessary for the maintenance of mucosal integrity. There are therefore numerous lines of evidence concerning the increased oxidative status in CD patients. However, oxidative stress is not limited to an increase in ROS production. It is also represented by a decrease in antioxidant defences which, as we shall see, is equally present in patients suffering from CD.

3.3.2 DECREASED ANTIOXIDANT DEFENCES

Various studies have reported altered levels of both enzymatic and low-molecular weight antioxidants in CD patients. It is worth noting that the colonic mucosa is endowed with low levels of endogenous antioxidant enzymes, which are mostly associated with epithelial cells, whereas the mucosal interstitium appears to be devoid of significant enzymatic defences against ROMs [218]. In fact, catalase and superoxide dismutase levels are less than 10% and GSH-Px levels less than 50% of those found in the liver. Therefore, the intestine is already less apt to defend itself against an oxidative attack. In addition, several groups have reported alterations in the activities of these enzymes in IBD. Decreased SOD activity was detected both in circulation and in the mucosa of CD patients. One study by Verspaget et al reported diminished SOD content in peripheral blood neutrophils from CD patients [206]. A few years later, the same group reported significantly lower Cu-Zn SOD concentrations in inflamed mucosal samples from CD patients compared to non-inflamed and control samples [219]. Another group later found decreased copper levels and Cu-Zn SOD activity in inflamed lesions from CD [213]. Taken together, these findings suggest a direct inverse relationship between inflammation and SOD.

Regarding GSH-Px activity, results seemed to be conflicting. While a few groups reported increased or unchanged levels, decreased plasma and erythrocyte GSH-Px activity was reported by most groups, particularly in active disease [209][220][221][222][223]. One group even found decreased GSH levels in both inflamed and non-inflamed mucosa and increased GSSG levels in inflamed mucosa from CD patients [224]. The same group detected significantly lower plasma cysteine levels in the presence of inflammation, cysteine being the rate-limiting precursor for GSH. Most importantly, they observed profound decreases in γ-glutamylcysteine synthetase (γGCS) and γ-glutamyl transferase (γGT) activity in the inflamed mucosa from CD patients as compared to healthy individuals. γGCS is the rate-limiting enzyme in GSH synthesis, and γGT is the enzyme that degrades luminal GSH and GSSG into amino acids for subsequent re-synthesis into GSH. Decreased activities of these enzymes predominantly contribute to intestinal GSH deficiency in both inflamed and non-inflamed ileum. In addition, the imbalance between GSH and GSSG levels during inflammation reflects a pro-oxidant shift of mucosal redox balance of glutathione in CD.

Besides endogenous antioxidants, several reports found decreased levels of dietary antioxidants in patients suffering from CD. The most prominent deficiencies were of ascorbic acid, vitamin A, selenium and β-carotene levels in the sera and plasma from CD patients [209][212][221][222][223][225]. In addition, a few groups reported decreases in α-carotene, β-cryptoxanthin, lycopene, magnesium, zinc, ubiquinol-10 and vitamin E levels in CD [213][226][227]. The most pronounced deficiencies were reflected in active disease. This suggests an increased consumption of antioxidants during free radical scavenging in response to ROS production.

In addition to measurements of individual vitamin and mineral antioxidants, one group went so far as to measure the total scavenging capacity of erythrocytes in active and quiescent CD, stating that the specific activities and concentrations of antioxidants such as enzymes and vitamins do not represent the total antioxidant status of erythrocytes [228]. They found an exhaustion of

scavenger capacity of erythrocytes, indicating serious deficiency in both phases of CD, even in patients supplemented with antioxidant therapy. Another group reported a marked loss of peroxyl scavenging capacity in mucosal preparations from the inflamed colon of CD patients [227].

Thus, in the setting of IBD, excess ROS production may overwhelm the exogenous and endogenous antioxidant defence systems, particularly in the presence of antioxidant deficiency. It can be concluded from the large body of scientific literature that chronic gut inflammation promotes an imbalance between pro-oxidant and antioxidant mechanisms. This undoubtedly has major implications in terms of intestinal immune function in CD, as pro-oxidant mechanisms are known to be involved in the activation of NF-κB, thereby mediating the transcription of several key cytokines and cell adhesion molecules.

3.3.3 ANTIOXIDANT THERAPY IN CD

The important role of reactive oxygen metabolites in intestinal injury is demonstrated by the beneficial effects of a variety of free radical scavengers, and by the fact that the aminosalicylates commonly used to treat IBD are also potent antioxidants. Various free radical scavengers have been tested in both animal models and in humans with IBD, with a few showing beneficial results. Agents blocking the release or effects of ROM, such as xanthine oxidase inhibitors, superoxide dismutase or its mimetic copper disopropyl salicylate (DIPS), catalase, the glutathione peroxidase mimetic PZ51, dimethyl sulfoxide (DMSO), and desferroxamine all decrease inflammation in animal or intestinal cell culture models of colitis. Superoxide dismutase proved to be successful in reducing the severity of acetic acid-, TNBS-, and carrageenan-induced colitis [229][230][231]. Both SOD and its mimetic DIPS provided significant protection against acetic acid-induced colitis [232]. Trials by the same group reported that catalase reduced chemically induced colitis as well. The addition of exogenous glutathione was found to provide rat small-intestinal epithelial cells with significant protection against oxidative injury [233]. Furthermore, the glutathione peroxidase mimetic PZ51 significantly reduced mucosal damage in a model of formyl-methionyl-

leucyl-phenylalanine (fMLP)-induced colitis. In the same series of experiments, similar results were obtained upon treatment with manganese-loaded desferroxamine, an iron chelator, and DMSO, a hydroxyl radical scavenger [234]. Finally allopurinol, a competitive inhibitor of xanthine oxidase, reduced carrageenan-induced injury in a rodent model of colitis [231].

One study of particular interest is that of Reimund and colleagues, who examined the effects of antioxidants on the *in vitro* production of inflammatory cytokines in CD [235]. Their experiments consisted in the addition of three antioxidants, namely butylated hydroxyanisole (BHA), tetrahydroxypapaveroline (THP), and nordihydroguaiaretic acid (NDGA) to PBMC and explant tissue cultures of inflamed mucosa from IBD patients. Their results show that all three antioxidants resulted in significant inhibition of IL-6 and IL-1 production by PBMC. Moreover, both BHA and THP significantly inhibited IL-1 production by CD inflamed mucosa. Their findings suggest that these antioxidants, in addition to their effects upon ROM production, may decrease cytokine production in IBD. In such a setting, antioxidants may be considered potential therapeutic tools in the treatment of CD.

As one can see, evidence for the beneficial role of antioxidants in animal or intestinal cell culture models is abundant. However, such results cannot be extrapolated to humans, and the evaluation of antioxidant therapy warrants further controlled trials in human subjects. In recent years, numerous attempts have been made to introduce antioxidant therapy as a major therapeutic concept in several diseases. Free radical scavengers were used as therapeutic agents following kidney and pancreas transplantation, in severely burned patients, in those with acute myocardial infarction, and in those suffering from diabetes mellitus type II. However, the outcomes of these studies were for the most part disappointing.

With regard to IBD, clinical data on trials with antioxidant therapy are scarce. The most prominent study was the one conducted by Emerit and colleagues, who administered bovine Cu-Zn SOD intramuscularly to 26 patients over the course of 8 years [236]. They reported good results in 77% of the patients over a 4-year follow-up period. Significant improvement of the clinical

situation was measured by the CD activity index, endoscopic demonstration of mucosal restoration and a shortening of relapse duration. In that study, the addition of desferroxamine seemed beneficial as well. However, subsequent trials of SOD therapy were abandoned due to the rapid elimination of SOD through the kidneys. No other controlled trials of antioxidant therapy, whether administered orally or rectally, have been published to date.

Nevertheless, a role for ROM in CD has been further supported by the discovery of antioxidant properties of aminosalicylates, drugs that are widely used in the treatment of IBD. Aminosalicylic compounds exert various anti-inflammatory effects, including the inhibition of lipoxygenase activity, of the cyclooxygenase pathway, of prostanoid metabolism, and of various functions of inflammatory cells. More importantly, a critical part of the anti-inflammatory activity of 5-aminosalicylic acid (5-ASA) seems to be its potent antioxidant activity [237]. Indeed, 5-ASA has potent SOD-like activity, is an effective scavenger of the hydroxyl radical, inhibits peroxyl radical-mediated and hemoglobin-catalysed lipid peroxidation, and scavenges hypochlorous acid [238][239][240]. Furthermore, the finding of 5-ASA metabolites identical to those formed by the reaction of 5-ASA with the hydroxyl radical in faeces from patients with IBD treated with sulfasalazine suggests that ROM scavenging is a clinically important mechanism of action in IBD [211].

In conclusion, oxidative stress seems to play a pivotal role in mediating tissue injury in the chronic intestinal inflammation characteristic of IBD (figure 3). There is an increased number of activated inflammatory cells in the mucosa of CD that are capable of respiratory bursts, releasing toxic ROM. Many groups reported DNA, protein and lipid oxidation products in CD. In addition, antioxidant defences are depleted in the mucosa and in the systemic circulation of many CD patients. Furthermore, one group demonstrated that NF-κB is oxidatively modulated in intestinal cells from CD patients, with a heightened degree of activation and cytokine secretion in those affected. Thus, people suffering from CD are oxidatively stressed, and this seems to aggravate their inflammatory state. Antioxidant therapy was successful in certain cases.

Moreover, drugs such as sulfasalazine and 5-ASA have potent antioxidant properties. Therefore, it is reasonable to conclude that oxidative stress is of major importance in the pathophysiology of CD, and that future studies will perhaps elucidate the mechanisms and pathways through which oxidative stress exerts its deleterious effects on the organism.

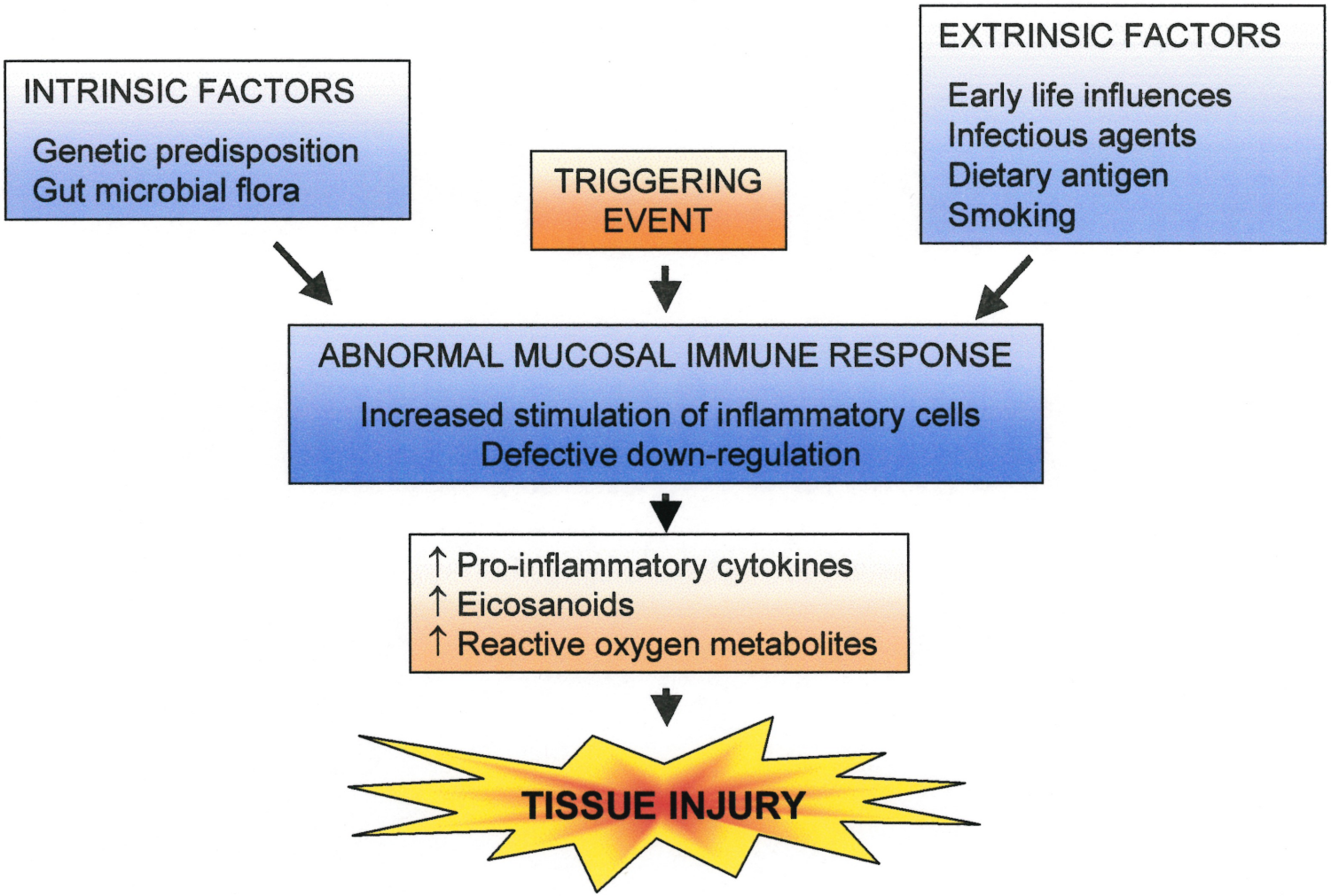

Figure 5 - Cascade of events leading to tissue injury in IBD.

RESEARCH PROJECT

Hypothesis and Objectives

Oxidative stress has been suggested to participate in the pathogenesis of many diseases, and the list of disorders in which radicals and oxidants have been implicated is still growing. In IBD, the implication of oxidative stress has been widely documented, particularly in CD.

Though the intestinal mucosa is constantly exposed to a variety of luminal antigen and oxidants, the exact effects of oxidative stress on intestinal epithelial cell antioxidant and inflammatory response have not been fully characterized. Our hypothesis is that exposure of intestinal epithelial cells to pro-oxidants will lead to alterations in cellular integrity and endogenous antioxidant functions, activation of the transcription factor NF-κB, and subsequent production of inflammatory mediators. The occurrence of increased levels of oxidants and of NF-κB activation in the mucosa of CD patients led us to consider this hypothesis.

The goal of our study was to evaluate the effects of the known pro-oxidant iron/ascorbate on lipid peroxidation, membrane integrity, endogenous antioxidant defense mechanisms, NF-κB activation, and inflammation mediator production in intestinal epithelial cells.

INFLAMMATORY REACTION WITHOUT ENDOGENOUS ANTIOXIDANT RESPONSE IN CACO-2 CELLS EXPOSED TO IRON/ASCORBATE-MEDIATED LIPID PEROXIDATION

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ABSTRACT

To characterize the role of intestinal epithelial cells in mucosal host defense, we have examined endogenous antioxidant reactivity and inflammatory response in Caco-2 cell line. When differentiated Caco-2 cells were incubated with iron/ascorbate for 1-24h, they exhibited increased MDA levels and decreased polyunsaturated fatty acid proportion in favor of saturated fatty acids. These modifications were accompanied with alterations in membrane fluidity and permeability. The oxidative stress did not induce changes in the antioxidant enzyme activity of superoxide dismutase, catalase, glutathione peroxidase and glutathione transferase, or in cellular glutathione content. However, iron/ascorbate-mediated lipid peroxidation promoted IκB degradation and NF-κB activation, as well as gave rise to interleukin-8, COX-2 and ICAM-1. These results support the importance of oxidant/antioxidant balance in the epithelial cell inflammatory response.

KEYWORDS

Oxidative stress; Membrane transport; NF-κB; COX-2; ICAM-1.

INTRODUCTION

Free radicals and oxidants are potentially deleterious molecules that have been implicated in the pathogenesis of many diseases [1][2]. In humans, normal biological activities and exogenous contributors lead to the formation of a large amount of highly reactive radicals every day [3]. These radicals can lead to the oxidation of DNA, proteins, lipids and other cellular constituents, which play a key role in the pathophysiology of various diseases, including inflammatory diseases and cancer [2]. Humans have developed antioxidant defenses and detoxification systems to protect against oxidative stress repercussions and to repair damaged biological components [3][4][5]. However, in many conditions, overproduction of radicals can overcome antioxidant defense systems and give rise to cellular oxidant burden, rendering tissues susceptible to oxidant-induced damage [6][7].

The lumen of the intestine is lined with a single layer of epithelial cells, mainly represented by absorptive columnar enterocytes. These cells are not only responsible for nutrient transport, but also actively participate in local epithelial immune response, which can enhance protection against microbial and dietary antigens [8]. With regards to the daily challenge by challenged by various diet-derived oxidants, the intestine is endowed with several defense mechanisms that preserve cellular integrity and tissue homeostasis [3][4][5][9][10]. It has become increasingly apparent that patients with Crohn's disease exhibit elevated free radical levels and improper antioxidant defense system [11][12][13][14][15][16]. In this debilitating illness, chronic oxidative stress produces marked elevations of DNA oxidation constituents and cell proteins, resulting in inflammation and tissue destruction [17][18][19]. Even if the etiology of Crohn's disease has yet to be fully elucidated, a close relationship has been noted between reactive species and the mucosal inflammatory process. However, the ability of intestinal cells to challenge

luminal oxidants by increasing endogenous defense mechanisms has not been thoroughly examined. Furthermore, the mechanisms by which oxidants contribute to inflammation are not entirely clear, but one proposed pathway may be through the activation of the transcription factor NF-κB, which initiates the expression of genes controlling several aspects of the inflammatory, immune and acute phase responses [20][21][22][23].

The aims of this study were, first, to determine whether intestinal cells preserve cellular integrity and appropriately respond to iron-catalyzed free radical-mediated lipid peroxidation by enhancing the activity of GSH peroxidase, GSSG reductase, GSH transferase, catalase and superoxide dismutase, as well as the levels of glutathione. Secondly, as an attempt to understand the oxidative modulation of inflammatory response, attention was directed toward the status of NF-κB activation and inflammation mediators. The well-characterized Caco-2 cell model was used in the present study to assess the effects of iron-induced lipid peroxidation on antioxidant enzyme systems and innate immune response.

MATERIALS AND METHODS

Cell Culture

Caco-2 cells (American Type Culture collection, Rockville, MD) were grown at 37°C in Minimum Essential Medium (MEM; GIBCO-BRL, Grand Island, NY), containing 1% penicillin/streptomycin, 1% MEM non-essential amino acids (GIBCO-BRL), supplemented with 10% decomplemented fetal bovine serum (FBS; Flow McLean, VA). Caco-2 cells (passages 30-40) were maintained with 5% CO_2 in T-75 cm^2 flasks (Corning Inc., Corning, NY). Cultures were split (1:6) when they reached 75-90% confluence, using 0.05% trypsin-o.5mM EDTA (GIBCO-BRL, Grand Island, NY). For individual experiments, cells were plated at a density of 1 x 10^6 cells/well on 23.1mm polyethylene terephthalate (PET) Falcon filter inserts with 0.4-μM pores (Becton Dickinson, NJ) in MEM supplemented with 5% FBS. The inserts were placed into six-well culture plates, permitting separate access to the upper and lower compartments of the monolayers. Cultures were maintained for 21 days, a period at which the Caco-2 cells are fully differentiated and suitable for studies on metabolic functions, such as lipid transport [24]. The medium was refreshed every second day.

Estimation of Lipid Peroxidation

Caco-2 cells were cultured in the presence or absence of Fe^{2+}/ascorbate (0.2μM : 2μM, Sigma, Montreal) for periods varying between 1 and 24 hours. The amount of free malondialdehyde (MDA) formed during the reaction was determined by HPLC, as we previously described [25]. Proteins were first precipitated with a 10% sodium tungstate (Na_2WO_4) solution (Aldrich Chemical Co., Milwaukee, WI). The protein-free supernatants were then reacted with an equivalent volume of 0.5% (W/V)

thiobarbituric acid solution (TBA; Sigma, Montreal) at 90°C for 60 minutes. After cooling to room temperature, the pink chromogene [$(TBA)_2$-MDA] was extracted with 1-butanol and dried over a stream of nitrogen at 37°C. The dry extract was then resuspended in a KH_2PO_4/methanol mobile phase (70:30, pH 7.0) before MDA detection by HPLC.

Effect of the BHT Antioxidant

In order to determine whether lipid peroxidation was responsible for the alterations caused by iron/ascorbate, the powerful antioxidant butylated hydroxytoluene (BHT; Sigma, Montreal) was added to the apical compartment, at a concentration of 150μM for 1h hour before the addition of the iron/ascorbate complex. BHT was dissolved in ethanol and control wells received an equal volume of the alcohol carrier (1% in the medium).

Brush Border Membrane Isolation and Cell Membrane Fluidity

Brush border membranes were purified from Caco-2 cell homogenates according to the method of Schmitz et al [26]. Briefly, the culture medium was removed and the cells attached to filters were rinsed twice with phosphate-buffered saline (PBS; GIBCO-BRL, Grand Island, NY). The cells were then scraped off and homogenized in Tris-HCl mannitol (2 mM HCl, 50 mM mannitol, pH 7.0). Following the addition of 10mM $MgCl_2$, the homogenates were centrifuged (7700 x g, 15 min, 4°C). The supernatants were subsequently centrifuged (20,000 x g, 30 min, 4°C) and the resulting pellets were used for the determination of fluidity.

Aliquots of the brush border membranes were diluted with 300 mM mannitol, 10 mM Tris-HEPES and 100mM KCl (pH 7.5). Fluidity was estimated by the incorporation

of the fluorescent probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluene-sulfonate, and measurement of polarization [25]. The final molar probe-to-lipid ratio was 1:1000. Fluorescence was measured in a spectrofluorometer at 25°C with polarization filters parallel and perpendicular to the excitation beam. Excitation was at 360 nm and emanation at 420 nm.

Permeability

Intestinal permeability was assessed with [^{51}Cr]-EDTA as described previously [27].

Fatty acid analysis

Cellular fatty acids were assayed by an improved method previously described by our group [28][29]. Briefly, each sample to be analyzed was subjected to direct trans-esterification and then injected into a gas chromatograph (model HP 5880; Hewlett-Packard, Rockville, MD) by using a 60-m fused silica capillary column with SP-2331.

Regulatory Antioxidant Enzyme Activity

The activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSSG-R) and glutathione transferase (GST) were measured in cell homogenates after incubation with pro-oxidants and/or antioxidants. Briefly, a substrate for each enzyme was added to the cell homogenates and rate of disappearance of the substrate was measured by spectrophotometry. Superoxide dismutase activity was determined essentially as described by Sun Y and colleagues [30]. The method for the other enzyme quantification was adapted from the protocol reported by Pippenger et al [31]. For catalase activity, cell homogenates were placed in a cuvette that received H_2O_2 (10mM) as a substrate and

absorbency was read at 240 nm for 3 min. For GSH-Px activity, cell homogenates were added to a PBS-based working solution (pH 7.0) containing 1mM GSH, 0.6 U GSSG-R and 100 μM NADPH with a small quantity of tert-butyl hydroperoxide (1.5 %) to initiate the reaction. For GST activity, cell homogenates were resuspended in a PBS buffer solution (pH 6.5) containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 15 mM GSH to initiate the reaction. For GSSG-R activity, cell homogenates were mixed with a reagent (1 mM GSSG, 100 μM NADPH, and 1% triton in PBS pH 7.6). For each of the glutathione enzymes, absorbency rate was measured in a spectrometer at 340 nm for 3 min.

Glutathione Levels

In order to measure total glutathione levels, Caco-2 cells were washed twice with PBS, scraped in a buffer (50 mM Tris-HCl, 0.1 mM EDTA-Na_2, 10 mM L-serine, and 20 mM Na-Borate, pH 7.6), and sonicated on ice. Proteins were precipitated by adding a 10% sulfosalicylic acid solution and centrifuging at 10,000 RPM for 4 min. The supernatants were collected in cuvettes to which were added NADPH as a substrate, dithionitrobenzene (DTNB), and a small quantity of exogenous GSSG-R. The rate of disappearance of NADPH was measured in a spectrometer at 412 nm during 8 minutes, and the concentration of total GSH was calculated using a standard curve.

Immunoblot Analysis of COX-2, IκB and ICAM-1

Following the incubation with the pro-oxidant and antioxidant, Caco-2 cells were washed twice with PBS, and scraped in 0.5 ml lysis buffer (50 mM Tris-HCl pH 7.5, 150mM NaCl, 5 mM EDTA, 0.1% sodium docedyl sulfate (SDS), 0.5% sodium desoxycholate, 1% Triton, 1mM phenylmethylsulfonyl fluoride, 1mM BHT and 1mM

pepstatin. Cells were sonicated and aliquots of cell homogenates were mixed with loading buffer (Bio-Rad, Hercules, CA). Proteins were separated by SDS-polyacrylamide gel electrophoresis and were transferred onto Hybond nitrocellulose membranes (Amersham Chemical Corp., Baie d'Urfé, PQ). Then, the latter were blocked 1 hour with 2% skim milk in Tris-buffer containing 0.05% Tween, and incubated with the appropriate polyclonal antibody diluted in 1% milk TBST. After washing with TBST, membranes were incubated with the appropriate IgG horseradish peroxidase-linked antibody. The primary antibodies used were rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA), and the secondary antibody was anti-rabbit IgG horseradish peroxidase-linked antibody (Biosource, Camarillo, CA). Detection was performed by using an enhanced chemiluminescence system for antigen-antibody complexes.

Nuclear Extraction and Immunoblot Analysis of NF-κB

Nuclear extracts were prepared in order to verify NF-κB protein expression by Western blotting. Cells were washed twice with 1mM PBS-EDTA, and left on ice for 4 minutes in a lysis buffer containing 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.4% Nonidet, 2 mM DTT and anti-proteases. Cells were then scraped and centrifuged for 5 minutes at 1500 x g at 4°C. The pellets were then washed with another buffer (same composition as the previous one, but without the Nonidet), and centrifuged again under the same conditions. The resulting pellets were then resuspended in 50 μL of final hypertonic lysis buffer (20 mM HEPES, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 EDTA, 20% glycerol, 2 mM 1,4-dithio-DL-treithol and anti-proteases), and left on ice for 1 hour, with occasional vortexing. They were then centrifuged for 10 min at 10,000 x g at 4°C, and

the supernatants were collected for protein analysis and Western blots. Nuclear extraction techniques were modified from Egan et al[32].

In order to determine NF-κB protein expression, nuclear extracts were used instead of whole cell homogenates. Immunoblotting techniques were described above. Nitrocellulose membranes were incubated in 0.5% milk containing NF-κB p65 goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA). After washing with TBST, membranes were incubated with anti-goat IgG horseradish peroxidase-linked antibody (Biosource, Camarillo, CA) in 1% milk. Results were obtained by chemiluminescence.

Determination of IL-8

The cellular pro-inflammatory chemokine, interleukin-8, was measured using an ELISA kit (BD Biosciences, Mississauga, ON), following the incubation of Caco-2 cells with BHT and/or iron/ascorbate.

Statistical Analysis

All values are expressed as the mean ± SEM. Data were analyzed by the one-way ANOVA and the two-tailed student's t-test. A p value of ≤ 0.05 was considered significant.

RESULTS

MDA production following iron/ascorbate treatment

Experiments were first performed to examine the effects of iron/ascorbate on lipid peroxidation in Caco-2 cells. Iron/ascorbate caused a dose-dependent MDA production (Fig. 1A). On the other hand, the incubation of Caco-2 cells with iron/ascorbate over various periods of time resulted in constant cellular lipid peroxidation (Fig. 1B). The addition of the antioxidant BHT at a concentration of 150 μM suppressed the production of MDA, providing direct evidence for the ability of the iron/ascorbate system to provoke profound lipid peroxidation.

Transepithelial resistance, permeability and membrane fluidity

The treatment of Caco-2 cells with iron/ascorbate did not affect the morphology of Caco-2 cells that displayed a high degree of polarization, brush border microvilli, tight junctions and interdigitations (results not shown). This was consistent with recovered high transpithelial cell monolayer resistance (Table 1) that represents an indication of cell confluence and monolayer integrity, as well as a measure of tight junction formation. Even the transport of [^{51}Cr]-EDTA, a sensitive tool for the estimation of paracellular permeability, was lightly increased in Caco-2 cells supplemented with iron/ascorbate (Table 1), which suggests that only few molecules diffuse to the basolateral side via the paracellular shunt pathway.

Subsequent experiments were carried out to determine the effects of iron/ascorbate on membrane fluidity, which is representative of the relative motional freedom of lipid molecules in the membrane bilayer. Fluidity was measured by the incorporation of the fluorescent probe DPH in apical membranes isolated from Caco-2 cells. A significant decrease was observed with iron/ascorbate treatment. Pre-

incubation with BHT prevented the fluidity decline observed with iron/ascorbate, which suggests the direct involvement of oxidative stress.

Fatty Acid Composition

In agreement with the membrane fluidity data, differences were observed in cellular fatty acid composition. Overall, polyunsaturated fatty acids (PUFA) were markedly decreased in Caco-2 cells exposed to iron/ascorbate (Table 2). As a result, the ratio of PUFA to saturated fatty acids (SFA) was significantly lower by 79.4% in iron/ascorbate-treated cells when compared with control cells. In particular, the ratio of eicosatrienoic acid (20:3ω9) arachidonic acid (20:4ω6), a commonly used index for EFA deficiency, was significantly higher in iron/ascorbate-treated cells. Besides, BHT was capable of normalizing the FA composition abnormalities. Taken together, these data suggest that iron/ascorbate-mediated lipid peroxidation is able to induce a fall in membrane fluidity, probably by attacking polyunsaturated fatty acid bonds.

Endogenous antioxidant defense

In order to examine whether alterations in oxygen radical metabolizing enzymes occur in Caco-2 cells when incubated with iron/ascorbate, the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase and glutathione reductase were measured. As illustrated in Figures 2 and 3, there were no significant changes in the activities of all the aforementioned enzymes following the addition of iron/ascorbate or BHT. The incapacity of enzymes to react is also reflected by the unmodified total glutathione levels.

Degradation of IκB and activation of NF-κB

In order to verify whether iron/ascorbate could induce an inflammatory response, the degradation of the inhibitory protein IκBα and activation of the transcription factor NF-κB were verified. Following the addition of iron/ascorbate, Caco-2 cell homogenates were analyzed by Western blot, and the expression of the 37kDa protein IκBα was evaluated. As shown in Figure 4A, cells treated with iron/ascorbate exhibit decreased quantities of IκBα, which could account for by its degradation in the cytosol and the release of NF-κB. In parallel, the translocation of NF-κB to the nucleus was evidenced by the increased levels of the p65 NF-κB sub-fraction in nuclear extracts from cells treated with iron/ascorbate (Fig. 4A). Both degradation of IκBα and nuclear translocation of NF-κB were inhibited by the addition of the antioxidant BHT, suggesting that the oxidative properties of iron/ascorbate contribute to the activation of this transcription factor. Therefore, the addition of a pro-oxidant such as iron/ascorbate to intestinal epithelial cells activates NF-κB, which could then lead to the transcription of genes that regulate inflammatory protein synthesis.

Expression of ICAM-1, COX-2 and IL-8

The protein expression of intracellular adhesion molecule-1 (ICAM-1) and cyclooxygenase-2 (COX-2) was analyzed in Caco-2 cells. These two proteins are known to have a central role in inflammatory response. The incubation of Caco-2 cells with iron/ascorbate upregulated the protein expression of ICAM-1 and COX-2 (Fig. 4B and 4C). In the presence of BHT, iron/ascorbate was not able to induce the expression of these two proteins. Finally, the exposure of Caco-2 cells to iron/ascorbate enhanced the secretion of IL-8 into the basolateral medium, whereas BHT resulted in the elimination of the increased iron/ascorbate-mediated IL-8 output (Fig. 5).

DISCUSSION

Reactive oxygen intermediates represent important mediators in the activation of inflammatory responses. In particular, chronic gut inflammation is characterized by the enhanced production of reactive oxygen metabolites. The main objective of our work was to examine how intestinal epithelial cells, endowed with several antioxidant systems, behave in front of injurious effects of oxidative stress. Our *in vitro* studies clearly established the ineptitude of Caco-2 cells to efficiently promote their endogenous antioxidant systems in order to neutralize, or at least attenuate, iron/ascorbate-mediated lipid peroxidation. Consequently, inflammatory mechanisms were triggered, including the activation of NF-κB and the increased levels of IL-8, ICAM-1 and COX-2.

Intestinal epithelial cells represent a physiological barrier for luminal antigens, microbes and toxins. They are also active participants in the gut immune response with regard to the numerous cytokine receptors on their membrane surface and their capacity to secrete cytokines, chemokines and leukocyte adhesion molecules [33][34][35]. Therefore, the disruption of this interface could have serious consequences on barrier and immune functions. In order to evaluate the effects of oxidative stress on antioxidant defenses and immune reactions, we used the human colon adenocarcinoma Caco-2 cell line. The observations of our studies confirm that, at confluence, these cells differentiate into a highly polarized monolayer and manifest many of the features of small intestinal cells. Furthermore, we previously reported that Caco-2 cells display important functional characteristics such as lipid transport, lipoprotein synthesis and apolipoprotein biogenesis, which were all affected by iron/ascorbate-mediated lipid peroxidation [24][36][37]. Therefore, we utilized this intestinal epithelial cell model in the current investigation together with the well-established

iron/ascorbate-generating oxidative stress [38][39]. As was shown by our data, the addition of this pro-oxidant resulted in increased lipid peroxidation in a dose-dependent fashion. Accordingly, treatment with BHT, a lipid-peroxide scavenger, inhibited iron/ascorbate-mediated lipid peroxidation, thus pointing out the direct implication of iron/ascorbate in MDA production. The occurrence of lipid peroxidation was accompanied with essential fatty acid deficiency, as well as changes in membrane fluidity and permeability. Thus, the integrity of intestinal epithelial barrier seems compromised under the iron/ascorbate pro-oxidant effect. Noteworthy are the observations related to levels of lipid peroxidation in the blood circulation and mucosal biopsies of CD patients [40][29]. Available data in the scientific literature have also reported increased intestinal permeability in patients with Crohn's disease [41]. It is therefore reasonable to propose that reactive oxygen species can contribute to mucosal barrier disruption, thereby allowing various luminal antigens, bacteria and toxins to reach the lamina propria, activate immune cells, and elicit an excessive inflammatory response.

Human tissues are equipped with powerful defense mechanisms capable of withstanding oxidative attacks and limiting the damage to organs. The intestine possesses numerous endogenous antioxidant enzymes: SOD removes superoxide anions; catalase and glutathione peroxidase eliminate peroxides; glutathione reductase converts oxidized glutathione back to its reduced form, and glutathione transferase transfers oxidized glutathione to the extracellular medium. Surprisingly, the activities of these enzymes remained unchanged despite the marked lipid peroxidation produced by iron/ascorbate, which may explain the membrane alterations reported herein. A progressive fall in enzyme activities and depletion of glutathione content was expected to occur before the onset of any damage to cell constituents and functions. However, no upregulation in these antioxidants was noted despite the persistent oxidative stimuli.

This interesting aspect of our work deserves further investigation in humans, and if it is confirmed, therapeutic manœuvres should be designed to enhance mucosal antioxidant defense that may be useful to tackle the negative effects of oxidative stress. It should be noted that most antioxidant enzymes are located in the epithelial cells, whereas the underlying lamina propria is practically devoid of antioxidant enzymes [42].

A potential connection between oxidative stress and pro-inflammatory gene expression has been established [20][21][43]. It was therefore reasonable to determine whether iron/ascorbate-mediated lipid peroxidation could elicit an inflammatory response in Caco-2 cells. NF-κB represents a family of transcription factors that serve as important regulators of the host immune and inflammatory responses. Under resting conditions, NF-κB is sequestered in the cytoplasm by an inhibitory protein known as IκB [44][45]. Following activation by a wide array of external stimuli, a cascade of events occur, resulting in the degradation of IKB and the release of NF-κB, which subsequently translocates to the nucleus and transcriptionally modulates cellular genes implicated in early immune, acute phase and inflammatory responses [46]. Our findings emphasize that the treatment of Caco-2 cells with iron/ascorbate induced the degradation of cytosolic IκB with the ensuing translocation of NF-κB to the nucleus. Furthermore, the presence of BHT inhibited the activation of NF-κB, pointing out that this powerful transcription factor is subject to redox regulation in Caco-2 cells. Increased activation of NF-κB has been reported in both macrophages and intestinal epithelial cells in patients with Crohn's disease [47][48]. Collectively, these data indicate that in addition to luminal antigens and toxins that are deleterious to intestinal epithelial cells, pro-oxidants can overcome cell antioxidant defense, destabilize cell integrity and activate pro-inflammatory transcription factors.

Activation of the transcription factor results in the induction of inflammatory genes leading to the protein expression of pro-inflammatory cytokines. We therefore decided to examine whether the exposure of Caco-2 cells to iron/ascorbate triggers IL-8, ICAM-1 and COX-2. IL-8 functions as a potent neutrophil chemoattratant and activator, whereas ICAM-1 is a strong cell surface adhesion molecule [49][50]. The incubation of Caco-2 cells with iron/ascorbate led to augmented cellular production of IL-8 and expression of ICAM-1. Surprisingly, BHT produced the same effect, which did not allow us to highlight whether iron/ascorbate *per se* or iron/ascorbate-mediated peroxidation provoked the elevation of these two pro-inflammatory agents. Additional work with other exogenous antioxidants is needed to elucidate this interesting aspect. As to the COX-2 isoenzyme, mounting evidence suggests its direct implication in the production of pro-inflammatory prostaglandins. In the present investigation, COX-2 was found to be induced by iron/ascorbate, whereas BHT was effective in returning COX-2 protein levels back to control values. These findings reflect the need to restore pro-oxidant/antioxidant balance in an attempt to reduce the impact of powerful pro-inflammatory agents such as COX-2.

In summary, our data support an oxidative role of iron ascorbate in cell integrity and inflammatory response. It may therefore initiate intestinal cell damage and aggravate ongoing injury, particularly because the gut is not equipped with adequate antioxidant defense. From the elucidation of the involved mechanisms will emerge ways to test their *in vivo* relevance and to identify potential targets for intervention.

REFERENCES

[1] Kehrer JP. Free radicals as mediators of tissue injury and disease. *Critical Reviews in Toxicology* 23: 21-48, 1993.

[2] Kehrer JP, and Smith C. Free radicals in biology: sources, reactivities and role in the etiology of human diseases. In: Balz Frei, editor. *Natural Antioxidants in Human Health and Disease*. Academic Press, pp. 25-62, 1994.

[3] Halliwell B. Free radicals and antioxidants: a personal view. *Nutrition Reviews* 52: 253-65, 1994.

[4] Briviba K and Sies H. Nonenzymatic antioxidant defense systems. In: Balz Frei, editor. *Natural Antioxidants in Human Health and Disease*. Academic Press, pp. 107-128, 1994.

[5] Di Mascio P, Murphy ME, and Sies H. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. *The American Journal of Clinical Nutrition* 53: 194S-200S, 1991.

[6] Cochrane CG. Cellular injury by oxidants. *The American Journal of Medicine* 91: 23S-30S, 1991.

[7] Otamiri T. Oxygen radicals: their role in selected gastrointestinal disorders. *Digestive Diseases* 9: 133-41, 1991.

[8] Dionne S, Ruemmele FR, and Seidman EG. Immunopathogenesis of inflammatory bowel disease: role of cytokines and immune cell-enterocyte interactions, pp 41-57. In: Inflammatory bowel diseases. Bristan BR, Walker-Smith JA, eds. *Nestlé Nutrition Workshop Series* vol. 2. Karker, Switzerland, 1999.

[9] Cross CE, Halliwell B, and Allen A. Antioxidant protection: a function of tracheobronchial and gastrointestinal mucus. *Lancet* 1: 1328-30, 1984.

[10] Parks DA. Oxygen radicals: mediators of gastrointestinal pathophysiology. *Gut* 30: 293-8, 1989.

[11] Keshavarzian A, Sedghi S, Kanofsky J, List T, Robinson C, Ibrahim C, and Winship D. Excessive production of reactive oxygen metabolites by inflamed

colon: analysis by chemiluminescence probe. *Gastroenterology* 103: 177-85, 1992.

[12] Simmonds NJ, Allen RE, Stevens TR, Van Someren RN, Blake DR, and Rampton DS. Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. *Gastroenterology* 103: 186-96, 1992.

[13] Mulder TPJ, Verspaget HW, Janssens AR, de Bruin PAF, Pena AS, and Lamers CBHW. Decrease in two intestinal copper/zinc containing proteins with antioxidant function in inflammatory bowel disease. *Gut* 32: 1146-50, 1991.

[14] Hoffenberg EJ, Deutsch J, Smith S, and Sokol RJ. Circulating antioxidant concentrations in children with inflammatory bowel disease. *The American Journal of Clinical Nutrition* 65: 1482-8, 1997.

[15] Grisham MB. Oxidants and free radicals in inflammatory bowel disease. Lancet 344: 859-61, 1994.

[16] Weiss SJ. Tissue destruction by neutrophils. *The New England Journal of Medicine* 320: 365-76, 1989.

[17] Lih-Brody L, Powell SR, Collier KP, Reddy GM, Cerchia R, Kahn E, Weissman GS, Katz S, Floyd RA, McKinley MJ, Fisher SE, and Mullin GE. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Digestive Diseases and Sciences* 41: 2078-86, 1996.

[18] McKenzie SJ, Baker MS, Buffinton GD, and Doe WF. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *The Journal of Clinical Investigation* 98: 136-41, 1996.

[19] D'Odorico A, Bortolan S, Cardin R, D'Inca R, Martines D, Ferronato A, and Sturniolo GC. Reduced plasma antioxidant concentrations and increased oxidative DNA damage in inflammatory bowel disease. *The Scandinavian Journal of Gastroenterology* 36: 1289-94, 2001.

[20] Sen CK and Packer L. Antioxidant and redox regulation of gene transcription. *FASEB Journal* 10: 709-20, 1996.

[21] Schreck R, Rieber P, and Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO Journal* 10: 2247-58, 1991.

[22] Bonizzi G, Piette J, Schoonbroodt S, Greimers R, Havard L, Merville MP, and Bours V. Reactive oxygen intermediate-dependent NF-kappaB activation by interleukin-1beta requires 5-lipoxygenase or NADPH oxidase activity. *Molecular and Cellular Biology* 19: 1950-60, 1999.

[23] Natarajan R, Ghosh S, Fisher BJ, Diegelmann RF, Willey A, Walsh S, Graham MF, and Fowler AA 3rd. Redox imbalance in Crohn's disease intestinal smooth muscle cells causes NF-kappaB-mediated spontaneous interleukin-8 secretion. *The Journal of Interferon and Cytokine Research* 21: 349-59, 2001.

[24] Levy E, Mehran M, and Seidman E. Caco-2 cells as a model for intestinal lipoprotein synthesis and secretion. *FASEB journal* 9: 626-35, 1995.

[25] Brunet S, Thibault L, Delvin E, Yotov W, Bendayan M, and Levy E. Dietary iron overload and induced lipid peroxidation are associated with impaired plasma transport and hepatic sterol metabolism in rats. *Hepatology* 29: 1809-17, 1999.

[26] Schmitz J, Preiser H, Maestracci D, Ghosh BK, Cerda JJ, and Crane RK. Purification of the human intestinal brush border membrane. *Biochimica et Biophysica Acta* 323: 98-112, 1973.

[27] Baker SS and Baker RD. Caco-2 cell metabolism of oxygen-derived radicals. *Digestive Diseases and Sciences* 38: 2273-80. 1993.

[28] Lepage G, Levy E, Ronco N, Smith L, Galeano N and Roy CC. Direct transesterification of plasma fatty acids for the diagnosis of essential fatty acid deficiency in cystic fibrosis. *The Journal of Lipid Research* 30: 1483-90, 1989.

[29] Levy E, Rizwan Y, Thibault L, Lepage G, Brunet S, Bouthillier L, and Seidman E. Altered lipid profile, lipoprotein composition, and oxidant and antioxidant status in pediatric Crohn disease. *The American Journal of Clinical Nutrition* 71: 807-15, 2000.

[30] Sun Y, Oberley LW, and Li Y. A simple method for clinical assay of superoxide dismutase. *Clinical Chemistry* 34(3): 497-500, 1988.

[31] Pippenger CE, Browne RW, and Armstrong D. Regulatory antioxidant enzymes, pp 299-313. In: Free radical and antioxidant protocols. Armstrong D, ed. *Methods in Molecular Biology*, vol. 108. Humana Press, New Jersey, 1998.

[32] Egan LJ, Mays DC, Huntoon CJ, Bell MP, Pike MG, Sandborn WJ, Lipsky JJ, and McKean DJ. Inhibition of interleukin-1-stimulated NF-kappaB RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity. *The Journal of Biological Chemistry* 274: 26448-53, 1999.

[33] Yang SK, Eckmann LG, Panja J, and Kagnoff MF. Differential and regulated expression of C-X-C, C-C and C-chemokines by human colon epithelial cells. *Gastroenterology* 113: 1214-23, 1997.

[34] Dwinell MB, Eckmann L, Leopard JD, Varki NM, and Kagnoff MF. Chemokine receptor expression by human intestinal epithelial cells. *Gastroenterology* 117: 359-367, 1999.

[35] Huang G, Eckmann L, Savidge T and Kagnoff M. Infection of human intestinal epithelial cells with invasive bacteria upregulates apical intercellular adhesion molecule-1 (ICAM)-1) expression and neutrophil adhesion. *The Journal of Clinical Investigation* 98: 572-83, 1996.

[36] Levy E and Bendayan M. Use of immunoelectron microscopy and intestinal models to explore the elaboration of apolipoproteins required for intraenterocyte lipid transport. *Microscopy Research and Technique* 49: 374-82, 2000.

[37] Courtois F, Suc I, Garofalo C, Ledoux M, Seidman E, and Levy E. Iron-ascorbate alters the efficiency of Caco-2 cells to assemble and secrete lipoproteins. *The American Journal of Physiology: Gastrointestinal and Liver Physiology* 279: G12-9, 2000.

[38] Welch KD, Davis TZ, Van Eden ME, Aust SD. Deleterious iron-mediated oxidation of biomolecules(1) (6). *Free Radical Biology and Medicine* 32: 577-83, 2002.

[39] Brunet S, Thibault L, Lepage G, Seidman EG, Dube N, and Levy E. Modulation of endoplasmic reticulum-bound cholesterol regulatory enzymes by iron/ascorbate-mediated lipid peroxidation. *Free Radical Biology and Medicine* 28: 46-54, 2000.

[40] Ahnfelt-Ronne I, Nielsen OH, Christensen A, Langholz E, Binder V, and Riis P. Clinical evidence supporting the radical scavenger mechanism of 5-aminosalicylic acid. *Gastroenterology* 98: 1162-9, 1990

[41] DeMeo MT, Mutlu EA, Keshavarzian A, and Tobin MC. Intestinal permeation and gastrointestinal disease. The Journal of Clinical Gastroenterology 34: 385-96, 2002.

[42] Grisham MB, MacDermott RP, and Deitch EA. Oxidant defense mechanisms in the human colon. Inflammation 14(6): 669-80, 1990.

[43] Remick DG, and Villarete L. Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates. *The Journal of Leukocyte Biology* 59: 471-5, 1996.

[44] Thanos D and Maniatis T. NF-κB: a lesson in family values. *Cell* 80: 529-32, 1995.

[45] Finco TS, and Baldwin AS. Mechanistic aspects of NF-κB regulation: the emerging role of phosphorylation and proteolysis. *Immunity* 3: 263-72, 1995.

[46] Barnes PJ, and Karin M. Nuclear factor-B, a pivotal transcription factor in chronic inflammatory diseases. *The New England Journal of Medicine* 336: 1066-71, 1997.

[47] Podolsky DK. Inflammatory Bowel Disease. *The New England Journal of Medicine* 325: 928-37, 1991.

[48] Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, Knuechel R, Baeuerle PA, Scholmerich J, and Gross V. Nuclear factor kappa B is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology* 115: 357-69, 1998.

[49] Kunkel SL, Strieter RM, Chensue SW, Basha M, Standiford T, Ham J, Remick DG. Tumor necrosis factor-alpha, interleukin-8 and chemotactic cytokines. *Progress in Clinical and Biological Research* 349:433-44, 1990.

[50] Hayflick JS, Kilgannon P, and Gallatin WM. The intercellular adhesion molecule (ICAM) family of proteins. New members and novel functions. *Immunology Research* 17: 313-27, 1998.

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FIGURE LEGENDS

Figure 1: Lipid peroxidation as a function of time and concentration of iron/ascorbate. Differentiated Caco-2 cells were incubated with different concentrations of iron/ascorbate (A) or with 200 μM iron/ascorbate at 1, 2, 4, 6 and 8 hours (B). Then, MDA cell content was determined as described in Materials and Methods. BHT was added in some experiments at the concentration of 150 μM to neutralize the oxidative stress. Results shown are means ± SEM for 4 separate experiments, each carried out in triplicate. CTR, control; Fe, iron/ascorbate; BHT, butyrated hydroxytoluene.

* $p < 0.05$ vs. control.

Figure 2: Antioxidant activity as a function of iron/ascorbate concentration. The activity of superoxide dismutase, catalase and glutathione peroxidase was quantified as described in Materials and Methods. Results are expressed as the mean ± SEM of 4 separate experiments, each performed in triplicate.

Figure 3: Antioxidant activity of Caco-2 cells as a function of time. The activity of superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase, glutathione reductase and total glutathione was measured following the incubation with 200μM iron/ascorbate (Fe), 150μM butylated hydroxytoluene (BHT) or without any agent (CTR). Results are

expressed as the mean ± SEM of 4 separate experiments, each performed in triplicate.

Figure 4: IκB degradation, NF-κB activation, ICAM-1 and COX-2. Differentiated Caco-2 cells were treated with 200μM iron/ascorbate (Fe), 150μM butylated hydroxytoluene (BHT), remained untreated (CTR) for 4 hours. Interleukin-1 (IL-1) was examined at 5ng/ml for 1 hour as a positive control. Then, cells were homogenized and aliquots were applied on SDS-PAGE and immunoblotted as described in Materials and Methods. Panels A, B and C are representative of two separate experiments.

Figure 5: Effect of iron/ascorbate on interleukin-8 production. Differentiated Caco-2 cells were incubated with 200μM iron/ascorbate (Fe), 150μM butylated hydroxytoluene (BHT) or remained without treatment (CTR). Interleukin-8 (IL-8) was determined by ELISA as described in Materials and Methods. Results shown are means ± SEM for 4 experiments.

* $p < 0.05$ vs. control.

TABLE 1: EFFECT OF IRON/ASCORBATE ON MEMBRANE FEATURES

Treatment	TER ($\Omega.cm^2$)	Permeability (dpm/mg protein)	Fluidity (1/depolarization)
Control	2987 ± 141	1417 ± 44	3.42 ± 0.21
BHT	3124 ± 156	1566 ± 39	3.48 ± 0.17
Fe	2899 ± 163	1832 ± 48 *	2.91 ± 0.13
Fe/ BHT	3012 ± 144	1539 ± 51	3.50 ± 0.14

Transepithelial resistance (TER), permeability and membrane fluidity were determined in Caco-2 cells following 8-hour incubation with 200μM iron/ascorbate (Fe) and/or 150μM butylated hydroxytoluene (BHT). As described in Materials and Methods, TER was measured using a millicel-ERS apparatus, permeability by quantifying [Cr^{51}]-EDTA and membrane fluidity by the incorporation of a fluorescent probe. Values represent mean ± SEM of 3 separate experiments.

* $p < 0.05$ vs. control

TABLE 2: FATTY ACIDS AND INDEX OF EFA DEFICIENCY IN CACO-2 CELLS

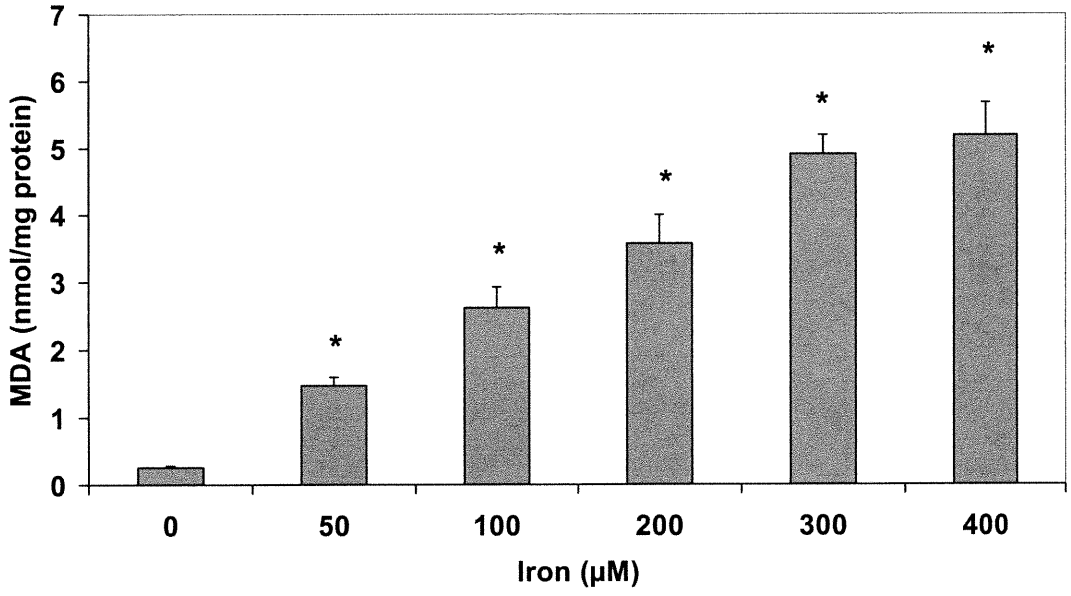
Fatty acids	Control	Fe	BHT	Fe/BHT
SFA	39.74 ± 0.42	42.63 ± 0.27	40.22 ± 0.39	37.32 ± 0.47
PUFA	13.48 ± 0.36	2.82 ± 0.17*	14.47 ± 0.44	12.95 ± 0.25
PUFA/ SFA	0.345 ± 0.112	0.059 ± 0.081*	0.371 ± 0.023	0.339 ± 0.177
20:3 ω 9/20:4 ω 6	0.093 ± 0.04	1.519 ± 0.125*	0.105 ± 0.04	0.104 ± 0.06

Caco-2 cells were cultured for 24 hours with 200µM iron/ascorbate (Fe) and/or 150µM butylated hudroxytoluene (BHT). At the end of the incubation period, cells were washed, scraped in PBS, centrifuged, and the resulting pellets were stored at -40 °C until analysis. Cellular fatty acid content was analyzed by GLC after direct trans-esterification. Results are expressed as percentage of total fatty acid content. On the basis of previous reports, criteria for essential fatty acid deficiency included cellular ratio of 20:3 ω 9/20:4 ω 6 superior to 0.2, in addition to decrease in total cell content of essential fatty acid. Data represents means ± SEM of 3 separate experiments. SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; EFA, essential fatty acids.

* $p < 0.005$ versus control, BHT, and Fe + BHT.

Figure 1

A


MDA (nmol/mg protein)
0
1
2
3
4
5
6
7
*
0
50
100
200
300
400
Iron (μM)

B

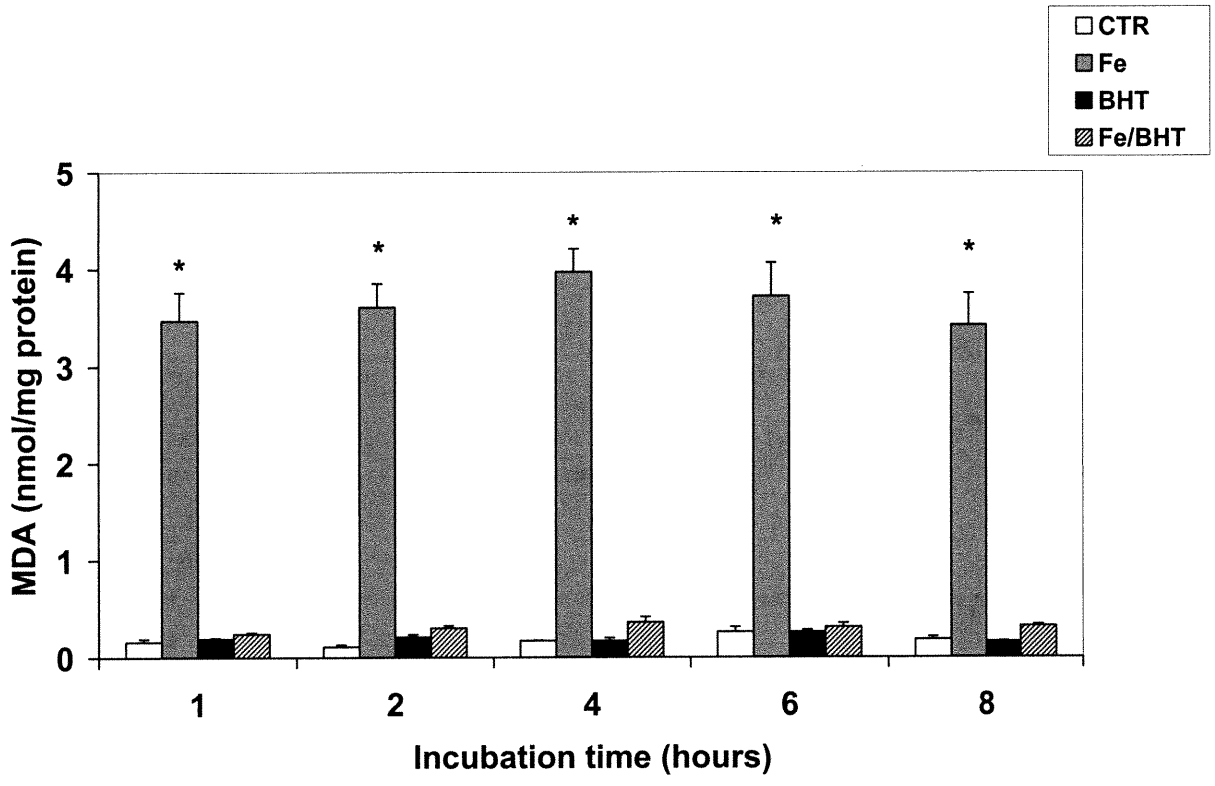

CTR
Fe
BHT
Fe/BHT
MDA (nmol/mg protein)
0
1
2
3
4
5
*
1
2
4
6
8
Incubation time (hours)

Figure 2

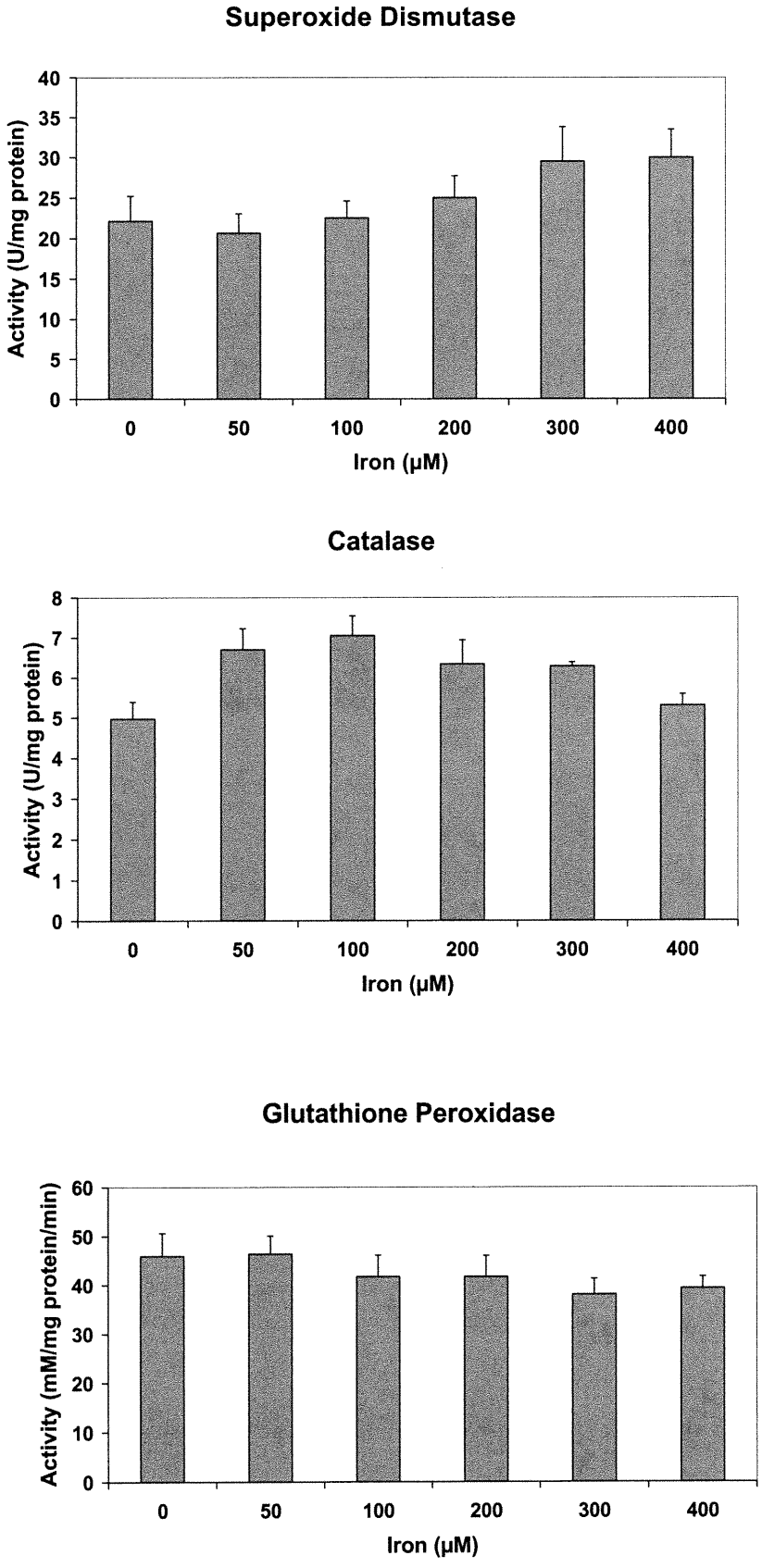

Superoxide Dismutase
Activity (U/mg protein)
0
5
10
15
20
25
30
35
40
0
50
100
200
300
400
Iron (μM)
Catalase
Activity (U/mg protein)
0
1
2
3
4
5
6
7
8
0
50
100
200
300
400
Iron (μM)
Glutathione Peroxidase
Activity (mM/mg protein/min)
0
10
20
30
40
50
60
0
50
100
200
300
400
Iron (μM)

Figure 3

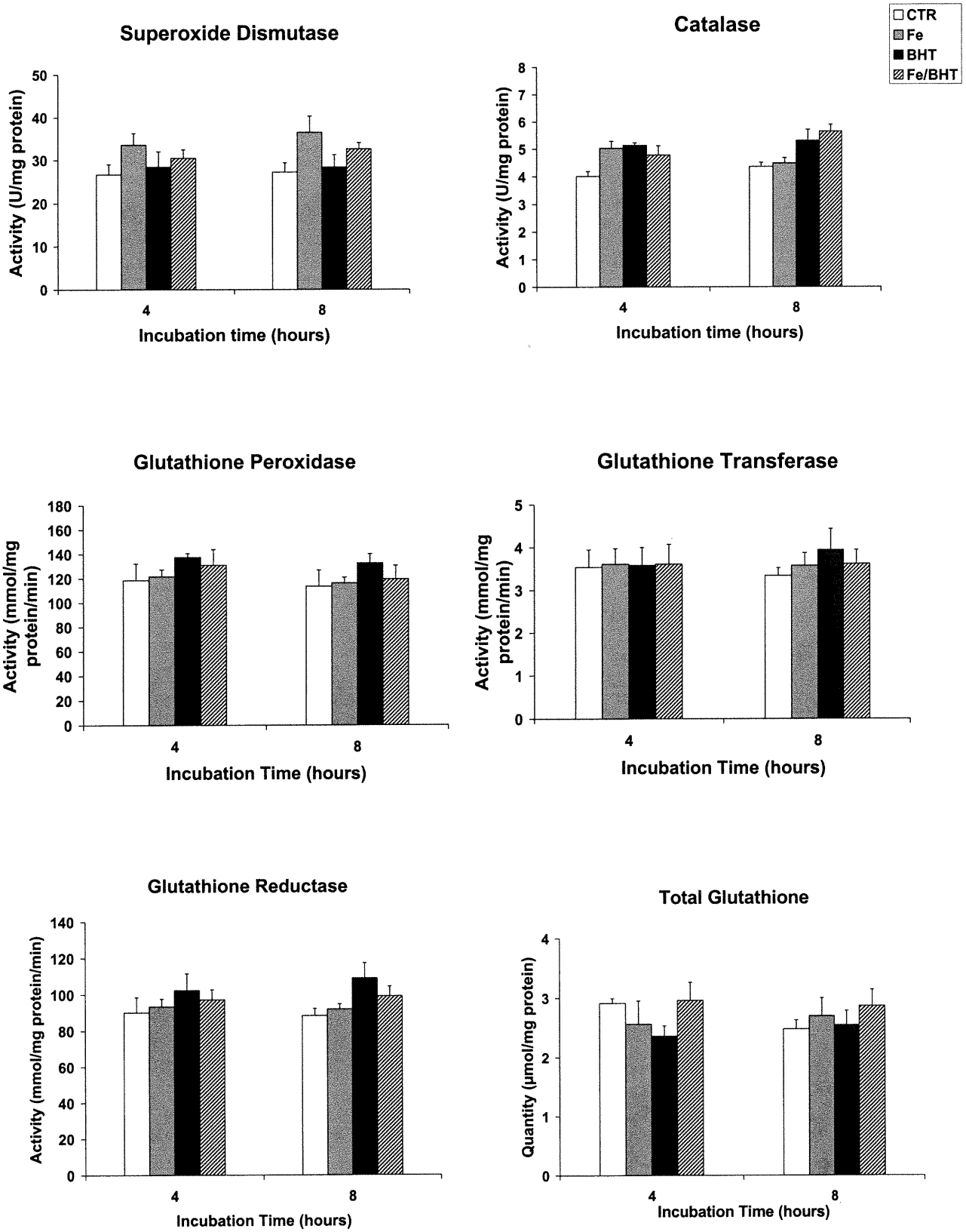

Superoxide Dismutase
Activity (U/mg protein)
Incubation time (hours)
Catalase
Activity (U/mg protein)
Incubation time (hours)
CTR
Fe
BHT
Fe/BHT
Glutathione Peroxidase
Activity (mmol/mg protein/min)
Incubation Time (hours)
Glutathione Transferase
Activity (mmol/mg protein/min)
Incubation Time (hours)
Glutathione Reductase
Activity (mmol/mg protein/min)
Incubation Time (hours)
Total Glutathione
Quantity (µmol/mg protein)
Incubation Time (hours)

Figure 4

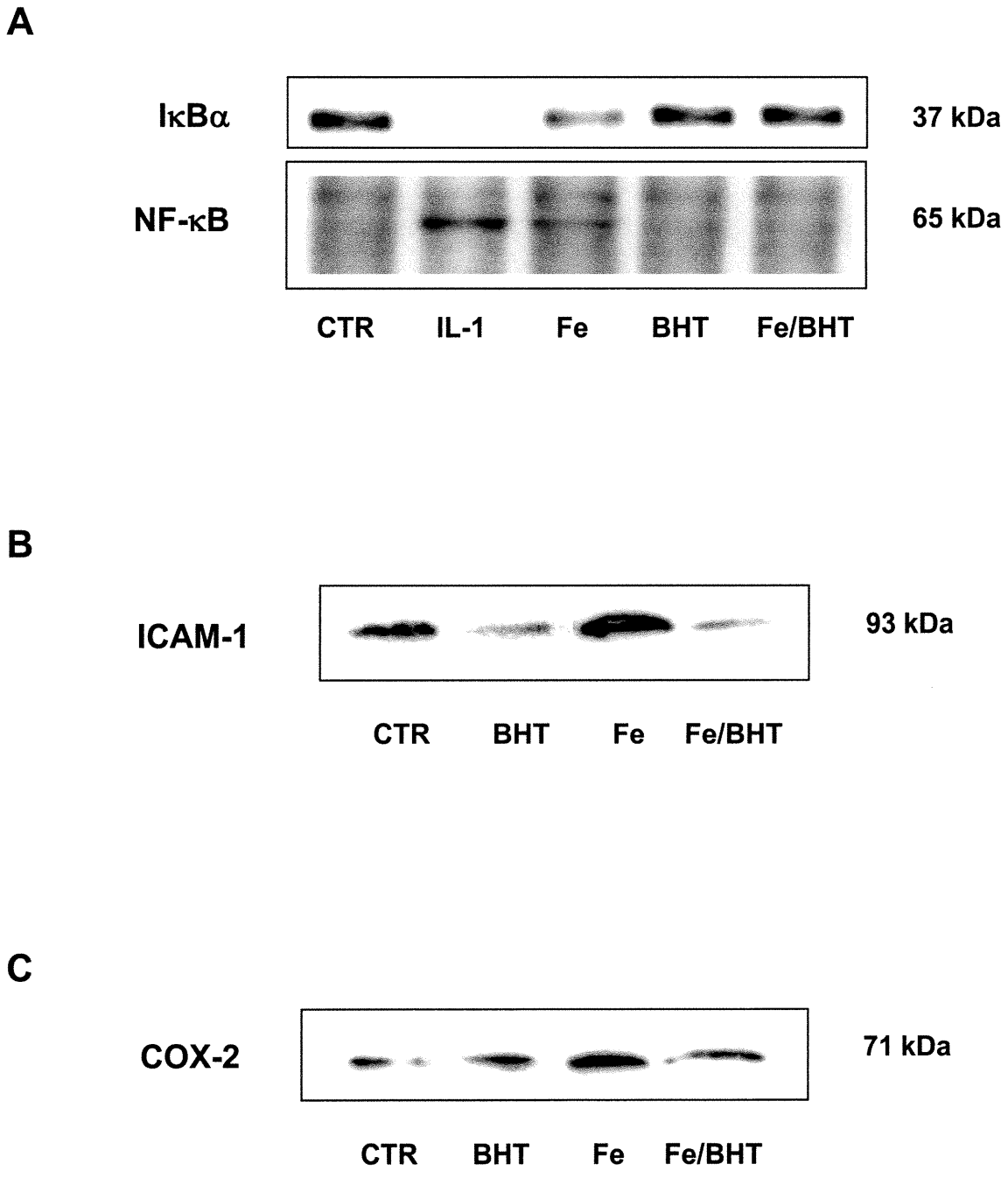
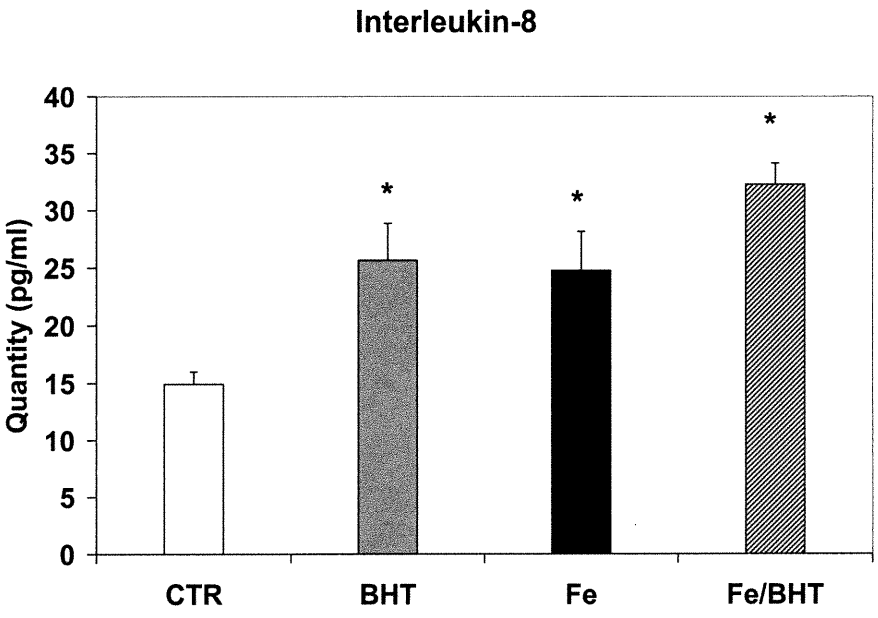

A
IκBα
37 kDa
NF-κB
65 kDa
CTR
IL-1
Fe
BHT
Fe/BHT
B
ICAM-1
93 kDa
CTR
BHT
Fe
Fe/BHT
C
COX-2
71 kDa
CTR
BHT
Fe
Fe/BHT

Figure 5


Interleukin-8
Quantity (pg/ml)
40
35
30
25
20
15
10
5
0
*
*
*
CTR
BHT
Fe
Fe/BHT

DISCUSSION

Many chronic inflammatory disorders, including CD, are associated with excessive production of reactive oxygen species (ROS) [241]. Under physiological conditions, numerous sources contribute to the formation of ROS, including phagocytes, bacterial and cellular oxidases, and unbound transition metals [169][242][243]. However, this production is increased during pathological disease states, and excessive ROS production can result in oxidative damage to a number of biological constituents [181][244]. The intestinal mucosa is one of the many targets of free radicals, and oxidant injury to the intestinal epithelium seems to be quite deleterious, particularly in the setting of IBD [244][245]. Intestinal epithelial cells represent a physiological barrier between luminal antigens, microbes and toxins, and the underlying mucosal immune system [246][247]. In addition, these cells are active participants in the gut immune response, considering that they display several cytokine receptors on their surface and are capable of secreting cytokines, chemokines and leukocyte adhesion molecules when primed [94][246]. Therefore, disruption of this system could have serious consequences on the barrier and immune functions of the intestinal mucosa.

In order to evaluate the effects of oxidative stress on mucosal integrity, antioxidant defences and immune reactions in intestinal epithelial cells, we employed the CACO-2 cell line as an *in vitro* model. At confluence, these cells differentiate into a highly polarized monolayer and display many of the features of small intestinal epithelial cells, including nutrient absorption, lipid synthesis and antioxidant enzyme activity [248][249][250][251]. The iron/ascorbate complex, a well-documented oxidant, was used to initiate free radical formation in our system. The quantities used were comparable to physiological concentrations of iron in the gut [252]. The choice of iron was based on the consideration that it is the most abundant transition metal in biological systems, and is integrally involved in many fundamental biochemical oxidation reactions [253]. Metal ions are usually sequestered by proteins under normal physiological conditions, but

are released from their chelators when the pH goes below 6 in the presence of inflammation, and are free to participate in Fenton-type reactions [243]. It is thus of interest that the mucosal content of iron in biopsies from inflamed CD lesions was found to be significantly higher than that of control tissue [213].

Our studies showed that the addition of the pro-oxidant complex resulted in an increase in membrane lipid peroxidation. This effect seemed to be dose-dependent, as was evidenced by the increasing production of MDA with increasing quantities of iron/ascorbate added. The addition of BHT, a lipid-peroxide scavenger, inhibited lipid peroxidation, thus proving the direct implication of our oxidant complex in disrupting the cellular redox balance. In our *in vitro* model, the occurrence of lipid peroxidation was accompanied by a decrease in membrane PUFA in favour of SFA levels, indicating essential fatty acid deficiency in cells exposed to oxidative stress.

Lipid peroxides are toxic and capable of damaging most body cells. Moreover, the occurrence of lipid peroxidation in biological membranes impairs membrane functioning and changes in fluidity [182]. Indeed, we found that the addition of a pro-oxidant resulted in a significant decrease in CACO-2 cell membrane fluidity, as well as a trend towards higher paracellular permeability. Pre-incubation with the antioxidant BHT corrected these disturbances, indicating that the alterations in membrane fluidity and paracellular permeability are induced by lipid peroxidation. This indicates that oxidative stress results in a decrease in the motional freedom of lipid molecules in the membrane bilayer, as well as an increase in the uptake of molecules through intercellular tight junctions. In other words, the integrity of the intestinal epithelial barrier is compromised under the effect of pro-oxidants. In patients with CD, increased levels of lipid peroxidation have been reported by several groups, both in the circulation and in mucosal tissue [209][210][211][212]. Direct evidence has been put forth that oxidant-induced injury to intestinal epithelial cells is increased in IBD [213][214]. In addition, several studies have reported increased intestinal permeability in CD patients [124][125]. It is suggested that the excessive ROS and free radicals found in the intestinal lumen of CD patients contribute to the disruption of the mucosal barrier. This would then allow luminal antigens,

bacteria or toxins to cross the mucosal barrier, activate immune cells, and elicit and perpetuate the excessive inflammatory response.

To determine whether intestinal epithelial cells are equipped to neutralise external sources of oxidative stress, we measured the activities of several endogenous antioxidant enzymes in CACO-2 cells. Several defence mechanisms limit the levels of reactive oxidants and the damage they inflict to tissues and organs. Among these are the endogenous antioxidant enzymes catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione transferase, and the key low-molecular weight antioxidant, glutathione. While SOD removes superoxide anions, CAT and GSH-Px are peroxide removing enzymes, with GSH-Px mostly dealing with lipid peroxides [171][172]. GSSG-reductase usually converts oxidized glutathione back to its reduced form, while GST transfers excess oxidized glutathione to the extracellular compartment [183]. It is important to note that in addition to its free radical scavenging properties, GSH serves as a substrate for GSH-Px, and as a result is oxidized to GSSG [183]. In all, endogenous antioxidants function to remove excess radicals from the cell in order to prevent a shift in the redox equilibrium. Upon the addition of iron/ascorbate, we expected the antioxidant enzymes to display a surge in activity, in order to control the oxidative attack, with an eventual return to normal activity levels. Surprisingly enough, the activities of these enzymes were unaltered in CACO-2 cells following the addition of either pro-oxidant and/or antioxidant, and remained stable over the ensuing 24 hours. It seems that these enzymes are unable to counter the oxidative attack that is initiated by iron/ascorbate, potentially leading to disruption of membrane functions, as we have reported here. It is important to note that the intestinal content of antioxidant enzymes is relatively low compared to organs such as the liver. Intestinal catalase and superoxide dismutase levels are less than 10% and glutathione peroxidase levels less than 50% of those found in the liver [218]. In addition, most of these antioxidant enzymes are located in the mucosal epithelium, with the underlying lamina propria practically devoid of this important defence system. Therefore, the epithelial compartment is comparatively better protected than the mucosal

interstitium, representing the main antioxidant defence against luminal oxidants. The finding of low levels of antioxidants in normal colonic mucosa – compared to hepatocytes – suggests that dietary oxidants in the lumen or activated inflammatory cells may easily overcome mucosal endogenous antioxidant defences [218]. If the mucosal enzymatic antioxidants are compromised, luminal oxidants have the potential to contribute to the pathogenesis of mucosal injury in IBD by causing epithelial cell injury, leading to mucosal erosions and alterations in epithelial permeability [244][252]. Indeed, our experiments demonstrated that the endogenous antioxidants of intestinal epithelial cells lend little protective resistance in the presence of an external source of oxidative stress. This means that the oxidants are free to damage several biological constituents of the cell, as represented by the decrease in membrane fluidity and increase in trans-epithelial permeability.

Once we established that endogenous antioxidants did not neutralise the oxidative attack, we proceeded to verify whether the oxidants could elicit an inflammatory response in CACO-2 cells, and whether the NF-κB pathway is involved. NF-κB represents a family of transcription factors that serve as important regulators of the host immune and inflammatory responses. In unstimulated conditions, NF-κB is sequestered in the cytoplasm by an inhibitory protein known as IκB [108]. NF-κB can be activated by a wide array of agents, including cytokines and oxidants [112]. During an immune response, external stimuli activate a cascade of events that result in the degradation of IκB and the release of NF-κB, which subsequently translocates to the nucleus and transcriptionally regulates cellular genes implicated in early immune, acute phase, and inflammatory responses [110]. Our results show that the addition of iron/ascorbate induced the degradation of cytosolic IκB with the ensuing translocation of NF-κB to the nucleus. The addition of an antioxidant inhibited the activation of NF-κB, proving that its activation in CACO-2 cells is subject to redox modulation. Redox modulation of NF-κB has been reported in various experimental models, with the general consensus that NF-κB response to

oxidants is cell- and stimulus-dependent [189][190][197][200]. In CD, increased activation of NF-κB has been reported in both macrophages and intestinal epithelial cells [156]. Yet to our knowledge, only one group succeeded in demonstrating redox-mediated NF-κB activation in CD biopsies [201]. Thus, as confirmed by our results, oxidative stress represents an alternative pathway for the activation of NF-κB in the intestinal mucosa. In addition to luminal antigen and toxins that are deleterious to intestinal epithelial cells, oxidants present in the mucosa can overcome cell antioxidant defences, destabilize cell integrity, and activate transcription factors.

Activation of a transcription factor such as NF-κB normally results in the activation of the genes of a series of inflammatory mediators, resulting in a cascade of events aggravating the inflammatory status of the patient. We therefore decided to verify whether exposure to oxidative stress could induce an inflammatory response, represented by COX-2 activation, ICAM-1 expression and IL-8 secretion in our experimental model. COX-2 is an inducible enzyme that catalyzes the first two steps in the biosynthesis of prostaglandins (PGs) from the substrate arachidonic acid [254]. COX-2 activity is normally absent from cells, but its expression can be upregulated by pro-inflammatory cytokines, growth factors and tumor promoters [255][256]. Thus, most of the stimuli that induce COX-2 are associated with inflammation. NF-κB has been shown to control the induced transcription of the COX-2 gene, and COX-2 can regulate NF-κB activity through a positive or negative feedback loop as well [257][258][259]. In addition, oxidative stress has been shown to induce COX-2 expression in different experimental models [260][261]. In CD, upregulated COX-2 expression has been reported in mononuclear and epithelial cells, suggesting a critical role for COX-2 in the development or maintenace of the inflammatory disease state [262][263]. Our results showing an upregulation of COX-2 levels in the presence of iron/ascorbate, and its inhibiton by pre-incubation with BHT, suggest an important link between oxidative stress and the production of inflammatory mediators in intestinal epithelial cells.

ICAM-1 is a cell surface bound glycoprotein that mediates adhesion-dependant cell to cell interactions. ICAM-1 has a restricted tissue distribution and is constitutively expressed at low levels on sub-populations of hemopoietic cells, vascular endothelium, fibroblasts and certain epithelial cells [264]. Its expression is markedly up-regulated at sites of inflammation, particularly by pro-inflammatory cytokines and bacterial products such as LPS [265][266]. The gene promoter for ICAM-1 possesses binding sites for several transcription factors, including NF-κB, and ICAM-1 expression can be up-regulated by oxidative stress [267][268]. In CD, increased expression of ICAM-1 has been reported in endothelial cells, leukocytes and apical membranes of epithelial cells [269]. Our results document an increase in ICAM-1 expression on CACO-2 cells subjected to the pro-oxidant, with a return to normal levels following the addition of the antioxidant. Thus, intestinal epithelial cells exposed to oxidative stress display increased ICAM-1 on their surface, possibly to mediate leukocyte binding as part of the inflammatory response.

Interleukin-8 is a chemokine that is secreted by a wide variety of cell types, including endothelial and epithelial cells [270][271]. In the gastrointestinal tract, IL-8 is capable of promoting the migration of neutrophils and macrophages across endothelial surfaces into bowel mucosa and submucosa. IL-8 is regulated primarily at the level of gene transcription and its promoter region contains functional binding sites for the transcription factors NF-κB, C/EBP, and activator protein (AP)-1 [272][273]. In addition, IL-8 can be induced by oxidant stress and its expression can be inhibited by antioxidants [268][274][275]. In CD, increased IL-8 levels have been reported by several groups, and IL-8 is recognised as intimately involved in the pathogenesis of CD colitis [276][277][278]. One group in particular reported that IL-8 production in CD smooth muscle cells is mediated by redox activation of NF-κB, thus providing a direct link between oxidative stress and IL-8 secretion in the inflamed gut [201]. Our results show that the addition of iron/ascorbate induced a significant increase in IL-8 production by intestinal epithelial cells, subsequent to the activation of NF-κB, confirming previous studies on oxidant-mediated production of IL-8 in

intestinal mucosa. However, the addition of the antioxidant BHT did not decrease IL-8 secretion, but rather potentiated it.

Though surprising at first, these findings may be explained when the transcription factor AP-1 is taken into account. Indeed, unlike their effect on NF-κB, certain antioxidants induce the activation, and not the inhibition, of AP-1 [279]. As a matter of fact, the lipid peroxide quenchers BHT and BHA were both found to potentiate the activation of AP-1 [280][281]. Therefore, we can suggest that while BHT inhibits the activation of NF-κB, it exerts an opposite effect on AP-1, thereby increasing transcription of the IL-8 gene and resulting in increased IL-8 secretion by CACO-2 cells. However, this did not determine whether the increase in IL-8 induced by iron/ascorbate is due to the lipid peroxides produced or to the addition of iron/ascorbate itself. Further research using different antioxidants is warranted in order to clarify the mechanisms behind the induction of IL-8 by either iron/ascorbate or BHT.

In conclusion, our results prove that the exposure of intestinal epithelial cells to oxidant stress is deleterious not only because of the structural damage it causes, but also because it triggers an inflammatory response within the epithelial cells themselves. Indeed, CACO-2 cells exposed to a known pro-oxidant exhibited increased lipid peroxide production, essential fatty acid deficiency, compromised cell integrity, inadequate antioxidant defences, activation of NF-κB and production of inflammatory mediators. These findings are quite important because if validated *in vivo*, treatment strategies will have to be adapted in order to provide the intestinal mucosa with a better antioxidant defence system in order to handle the oxidant load that it is constantly exposed to. Co-culture experiments with intestinal epithelial cells and monocytes or neutrophils may be useful in order to provide further insight into the mechanisms involved in epithelial cell inflammatory response in the presence of oxidative stress.

REFERENCES

[1] Crohn BB, Ginsburg L, and Oppenheimer GD. Regional enteritis: a pathogenic and clinical entity. The Journal of the American Medical Association 99: 1323-9, 1932.

[2] Cosgrove M, Al-Atial RF, and Jenkens HR. The epidemiology of pediatric inflammatory bowel disease. Archives of Disease in Childhood 74(5): 460-1, 1996.

[3] Hassan K, Cowan F, and Jenkins HR. The incidence of childhood inflammatory bowel disease in Wales. The European Journal of Pediatrics 159(4): 261-3, 2000.

[4] Ekbom A, Helmick C, Zack M, and Adami HO. The epidemiology of inflammatory bowel disease: a large, population-based study in Sweden. Gastroenterolgy 100(2): 350-8, 1991.

[5] Tragnone A, Hanau C, Bazzocchi G, and Lanfranchi GA. Epidemiological characteristics of inflammatory bowel disease in Bologna, Italy – incidence and risk factors. Digestion 54(3): 183-188, 1993.

[6] Stowe SP, Redmond SR, Stormont JM, Shah AN, Chessin LN, Segal HL, and Chey WY. An epidemiologic study of inflammatory bowel disease in Rochester, New York. Hospital incidence. Gastroenterology 98(1): 104-10, 1990.

[7] Sandler RS. Epidemiology of IBD. In Targan SR, Shanalan F (eds). Inflammatory bowel disease from bench to bedside. Baltimore, Williams & Wilkins, pp. 5-30, 1994.

[8] Lashner BA. Epidemiology of inflammatory bowel disease. Gastroenterology Clinics of North America 24(3): 467-74, 1995.

[9] Russel M, and Stockbrügger R. Epidemiology of inflammatory bowel disease: an update. Scandinavian Journal of Gastroenterology 31(5): 417-27, 1996.

[10] Sonnenberg A, McCarty DJ, and Jacobsen SJ. Geographic variation of inflammatory bowel disease within the United States. Gastroenterology 100(1): 143-9, 1991.

[11] Sonnenberg A. Occupational distribution of IBD among German employees. Gut 31(9): 1037-40, 1990.

[12] Monk M, Mendeloff AI, Siegel CE, and Lilienfeld A. An epidemiological study of ulcerative colitis and regional enteritis among adults in Baltimore. II. Social and demographic factors. Gastroenterology 56(5): 847-57, 1969.

[13] Fireman Z, Grossman A, Lilos P, Eshchar Y, Theodor E, and Gilat T. Epidemiology of Crohn's disease in the Jewish population of central Israel, 1970-1980. The American Journal of Gastroenterology 84(3): 255-8, 1989.

[14] Roth MP, Petersen GM, McElree C, Feldman E, and Rotter JI. Geographic origins of Jewish patients with inflammatory bowel disease. Gastroenterology 97(4): 900-4, 1989.

[15] Sawczenko A, Sandhu BK, Logan RFA, Jenkins H, Taylor CJ, Mian S, and Lynn R. Prospective survey of childhood inflammatory bowel disease in the British Isles. The Lancet. 357(9262): 1093-4, 2001.

[16] Logan RFA. Inflammatory bowel disease incidence: up, down, or unchanged? Gut 42(3) 309-11, 1998.

[17] Hyams JS. Extraintestinal manifestations of inflammatory bowel disease in children. The Journal of Pediatric Gastroenterology and Nutrition 19(1): 7-21, 1994.

[18] O'Donoghue DP, and Dawson AM. Crohn's disease in childhood. Archives of Diseases in Children 52(8): 627-32, 1977.

[19] Polito JM, Childs B, Mellits ED, Tokayer AZ, Harris ML, and Bayless TM. Crohn's disease: influence of age at diagnosis on site and clinical type of disease. Gastroenterology 111(3): 580-6, 1996.

[20] Thearle M, Horlick M, Bilezikian JP, Levy J, Gertner JM, Levine LS, Harbison M, Berdon W, and Obersfield SE. Osteoporosis: an unusual presentation of childhood Crohn's disease. The Journal of Clinical Endocrinology and Metabolism 85(6): 2122-6, 2000.

[21] Harries AD, and Heatley RV. Nutritional disturbances in Crohn's disease. Postgraduate Medecine Journal 59(697): 690-697, 1983.

[22] Stokes MA. Crohn's disease and nutrition. The British Journal of Surgery 79(5):391-4, 1992.

[23] Kelly DG, and Flemming CR. Nutritional considerations in inflammatory bowel disease. Gastroenterology Clinics of North America 24(3): 597-611, 1995.

[24] Sofaer J. Crohn's disease: the genetic contribution. Gut 34(7): 869-71, 1993.

[25] Orholm M, Binder V, Sorensen TI, Rasmussen LP, and Kyvik KO. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. The Scandinavian Journal of Gastroenterology 35(10): 1075-81, 2000.

[26] Tysk C, Lindberg E, Jarnerot G, and Floderus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. Gut 29(3): 990-6, 1998.

[27] Satsangi J, Jewell DP, Rosenberg WM, and Bell JI. Genetics of inflammatory bowel disease. Gut 35(5): 696-700, 1994.

[28] Bayless TM, Tokayer AZ, Polito JM, Quaskey SA, Mellits ED, and Harris ML. Crohn's disease: concordance for site and clinical type in affected

family members - potential herditary influences. Gastroenterology 111(3): 573-9, 1996.

[29] Colombel JF, Grandbastien B, Gower-Rousseau C, Plegat S, Evrard JP, Dupas JL, Gendre JP, Modigliani R, Belaiche J, Hostein J, Hugot JP, van Kruiningen H, and Cortot A. Clinical characteristics of Crohn's disease in 72 families. Gastroenterology 111(3): 604-7, 1996.

[30] Kuster W, Pascoe C, Purrmann J, Funk S, and Majewski F. The genetics of Crohn disease: complex segregation analysis of a family study with 265 patients with Crohn disease and 5387 relatives. The American Journal of Medical Genetics 32(1): 105-8, 1989.

[31] Lee JC, Bridger S, McGregor C, Macpherson AJ, and Jones JE. Why children with inflammtory bowel disease are diagnosed at a younger age than their affected parent. Gut 44(6): 808-11, 1999.

[32] Hugot JP, Laurent-Puig P, Gower-Rousseau C, Olson JM, Lee JC, Beaugerie L, Naom I, Dupas JL, Van Gossum A, Orholm M, Bonaiti-Pellie C, Weissenbach J, Mathew CG, Lennard-Jones JE, Cortot A, Colombel JF, and Thomas G. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. Nature 379(6568): 821-3, 1996.

[33] Orholm M, Iselius L, Sorensen TI, Munkholm P, Langholz E, and Binder V. Investigation of inheritance of chronic inflammatory bowel diseases by complex segregation analysis. The British Medical Journal 306(6869): 20-4, 1993.

[34] Satsangi J, Parkes M, Louis E, Hashimoto L, Kato N, Welsh K, Terwilliger JD, Lathrop GM, Bell JI, and Jewell DP. Two-stage genome-widesearch in inflammatory bowel disease: evidence for susceptibility loci on chromosomes 3, 7 and 12. Nature Genetics 14(2): 199-202, 1996.

[35] Cho JH, Nicolae DL, Gold LH, Fields CT, LaBuda MC, Rohal PM, Pickles MR, Qin L, Fu Y, Mann JS, Kirschner BS, Jabs EW, Weber J, Hanauer SB, Bayless TM, and Brant SR. Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1p, 3q, and 4q: evidence for epistasis between 1p and IBD1. Proceedings of the National Academy of Science of the United States of America 95(13): 7502-9, 1998.

[36] Rioux JD, Silverberg MS, Daly MJ, Steinhart AH, McLeod RS, Griffiths AM, Green T, Brettin TS, Stone V, Bull SB, Bitton A, Williams CN, Greenberg GR, Cohen Z, Lander ES, Hudson TJ, and Siminovitch KA. Genomewide search in candian families with inflammatory bowel disease reveals novel susceptibilty loci. The American Journal of Human Genetics 66(6): 1863-70, 2000.

[37] Danze PM, Colombel JF, Jacquot S, Loste MN, Heresbach D, Ategbo S, Khamassi S, Perichon B, Semana G, Charron D, and Cezard JP. Association of HLA class II genes with susceptibility to Crohn's disease. Gut 39(1): 69-72, 1996.

[38] Toyoda H, Wang SJ, Yang HY, Redford A, Magalong D, Tyan D, McElree CK, Pressman SR, Shanahan F, Targan SR, et al. Distinct associations of HLA class II genes with inflammatory bowel disease. Gastroenterology 104(3): 741-8, 1993.

[39] Plevy SE, Targan SR, Yang H, Fernandez D, Rotter JI, and Toyoda H. Tumor necrosis factor microsatellites define a Crohn's disease-associated haplotype on chromosome 6. Gastroenterology 110(4): 1053-60, 1996.

[40] Louis E, Satsangi J, Roussomoustakaki M, Parkes M, Fanning G, Welsh K, and Jewell D. Cytokine gene polymorphisms in inflammatory bowel disease. Gut 39(5): 705-10, 1996.

[41] Parkes M, Satsangi J, and Jewell D. Contribution of the IL-2 and IL-10 genes to inflammatory bowel disease susceptibility. Clinical and Experimental Immunology 113(1): 28-32, 1998.

[42] Bioque G, Crusius JB, Koutroubakis I, Bouma G, Kostense PJ, Meuwissen SG, and Pena AS. Allelic polymorphism in IL-1 beta and IL-1 receptor antagonist (IL-1Ra) genes in inflammatory bowel disease. Clinical and Experimental Immunology 102(2): 379-83, 1995.

[43] Heresbach D, Alizadeh M, Dabadie A, Le Berre N, Colombel JF, Yaouanq J, Bretagne JF, and Semana G. Significance of interleukin-1beta and interleukin-1 receptor antagonist genetic polymorphism in inflammatory bowel diseases. The American Journal of Gastroenterology 92(7): 1164-9, 1997.

[44] Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, and Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411(6837): 599-603, 2001.

[45] Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, and Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 411(6837): 603-6, 2001.

[46] Hampe J, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, Frenzel H, King K, Hasselmeyer A, MacPherson AJ, Bridger S, van Deventer S, Forbes A, Nikolaus S, Lennard-Jones JE, Foelsch UR, Krawczak M, Lewis C, Schreiber S, and Mathew CG. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. The Lancet 357(9272): 1925-8, 2001.

[47] Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, and Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. The Journal of Biological Chemistry 276(7): 4812-8, 2001.

[48] Inohara N, Ogura Y, Chen FF, Muto A, and Nunez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. The Journal of Biological Chemistry 276(4): 2551-4, 2001.

[49] Somerville KW, Logan RF, Edmond M, and Langman MJ. Smoking and Crohn's disease. The British Medical Journal 289(6450): 954-5, 1984.

[50] Franceschi S, Panza E, La Vecchia C, Parazzini F, Decarli A, and Bianchi Porro G. Nonspecific inflammatory bowel disease and smoking. The American Journal of Epidemiology 125(3): 445-52, 1987.

[51] Silverstein MD, Lashner BA, Hanauer SB, Evans AA, and Kirsner JB. Cigarette smoking in Crohn's disease. The American Journal of Gastroenterology 84(1): 31-3, 1989.

[52] Persson PG, Ahlbom A, and Hellers G. Inflammatory bowel disease and tobacco smoke--a case-control study. Gut 31(12): 1377-81, 1990.

[53] Ashley MJ. Smoking and diseases of the gastrointestinal system: an epidemiological review with special reference to sex differences. The Canadian Journal of Gastroenterology 11(4): 345-52, 1997.

[54] Sutherland LR, Ramcharan S, Bryant H, and Fick G. Effect of cigarette smoking on recurrence of Crohn's disease. Gastroenterology 98(5 pt 1): 1123-8, 1990.

[55] Cottone M, Rosselli M, Orlando A, Oliva L, Puleo A, Cappello M, Traina M, Tonelli F, and Pagliaro L. Smoking habits and recurrence in Crohn's disease. Gastroenterology 106(3): 643-8, 1994.

[56] Cosnes J, Beaugerie L, Carbonnel F, and Gendre JP. Smoking cessation and the course of Crohn's disease: an intervention study. Gastroenterology 120(5): 1093-9, 2001.

[57] Wakefield AJ, Sankey EA, Dhillon AP, Sawyerr AM, More L, Sim R, Pittilo RM, Rowles PM, Hudson M, and Lewis AA, et al. Granulomatous vasculitis in Crohn's disease. Gastroenterology 100(5 pt 1): 1279-87, 1991.

[58] Martini GA, and Brandes JN. Increased consumption of refined carbohydrates in patients with Crohn's disease. Klin Wocheschr 54(8): 367-71, 1976.

[59] Thornton JP, Emmett PM, and Heaton KW. Diet and Crohn's disease: characteristics of the pre-illness diet. The British Medical Journal 2(6193): 762-4, 1979.

[60] Katschinski B, Logan RF, Edmond M, and Langman MJ. Smoking and sugar intake are separate but interactive risk factors in Crohn's disease. Gut 29(9): 1202-6, 1988.

[61] Shoda R, Matsueda K, Yamato S, and Umeda N. Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. The American Journal of Clinical Nutrition 63(5): 741-5, 1996.

[62] Reif S, Klein I, Lubin F, Farbstein M, Hallak A, and Gilat T. Pre-illness dietary factors in inflammatory bowel disease. Gut 40(6): 754-60, 1997.

[63] Wurzelmann JI, Lyles CM, and Sandler RS. Childhood infections and the risk of inflammatory bowel disease. Digestive Diseases and Science 39(3): 555-60, 1994.

[64] Ekbom A, Adami HO, Helmick CG, Jonzon A, and Zack MM. Perinatal risk factors for inflammatory bowel disease: a case-control study. The American Journal of Epidemiology 132(6): 1111-9, 1990.

[65] Van Kruiningen HJ, Colombel JF, Cartun RW, Whitlock RH, Koopmans M, Kangro HO, Hoogkamp-Korstanje JA, Lecomte-Houcke M, Devred M, Paris JC, et al. An in-depth study of Crohn's disease in two French families. Gastroenterology 104(2): 351-60, 1993.

[66] Montgomery SM, Morris DL, Pounder RE, and Wakefield AJ. Paramyxovirus infections in childhood and subsequent inflammatory bowel disease. Gastroenterology 116(4): 796-803, 1999.

[67] Van Kruiningen HJ, Mayo DR, Vanopdenbosch E, Gower-Rousseau C, Cortot A, and Colombel JF. Virus serology in familial Crohn disease. The Scandinavian Journal of Gastroenterology 35(4): 403-7, 2000.

[68] Sartor RB. Microbial factors in the pathogenesis of Crohn's disease, ulcerative colitis and experimental intestinal inflammation. In Kirsner JB and Shorter RJ (eds). Inflammatory Bowel Disease, 4th edition. Baltimore, Wiliams and Wilkins, 96-104, 1995.

[69] Wakefield AJ, Pittilo RM, Sim R, Cosby SL, Stephenson JR, Dhillon AP, and Pounder RE. Evidence of persistent measles virus infection in Crohn's disease. The Journal of Medical Virology 39(4): 345-53, 1993.

[70] Ekbom A, Wakefield AJ, Zack M, and Adami HO. Perinatal measles infection and subsequent Crohn's disease. The Lancet 344(8921): 508-10, 1994.

[71] Thompson NP, Montgomery SM, Pounder RE, and Wakefield AJ. Is measles vaccination a risk factor for inflammatory bowel disease? The Lancet 345(8957): 1071-4, 1995.

[72] Haga Y, Funakoshi O, Kuroe K, Kanazawa K, Nakajima H, Saito H, Murata Y, Munakata A, and Yoshida Y. Absence of measles viral genomic sequence in intestinal tissues from Crohn's disease by nested polymerase chain reaction. Gut 38(2): 211-5, 1996.

[73] Greenfield SM, Punchard NA, Teare JP, and Thompson RP. Review article: the mode of action of the aminosalicylates in inflammatory bowel disease. Alimentary Pharmacology and Therapeutics 7(4): 369-83, 1993.

[74] Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clinical Science 94(6): 557-72, 1998.

[75] Ferguson A, Campieri M, Doe W, Persson T, and Nygard G. Oral budesonide as maintenance therapy in Crohn's disease--results of a 12-

month study. Global Budesonide Study Group. Alimentary Pharmacology and Therapeutics 12(2): 175-83, 1998.

[76] Stein RB, and Lichtenstein GR. Medical therapy for Crohn's disease: the state of the art. Surgical Clinics of North America 81(1): 71-101, 2001.

[77] Present DH, Korelitz BI, Wisch N, Glass JL, Sachar DB, and Pasternack BS. Treatment of Crohn's disease with 6-mercaptopurine. A long-term, randomized, double-blind study. The New England Journal of Medecine 302(18): 981-7, 1980.

[78] Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF, and Rutgeerts PJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. The New England Journal of Medecine 337(15): 1029-35, 1997.

[79] Rutgeerts P, D'Haens G, Targan S, Vasiliauskas E, Hanauer SB, Present DH, Mayer L, Van Hogezand RA, Braakman T, DeWoody KL, Schaible TF, and Van Deventer SJ. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. Gastroenterology 117(4): 761-9, 1999.

[80] Sandborn WJ, Feagan BG, Hanauer SB, Present DH, Sutherland LR, Kamm MA, Wolf DC, Baker JP, Hawkey C, Archambault A, Bernstein CN, Novak C, Heath PK, and Targan SR. An engineered human antibody to TNF (CDP571) for active Crohn's disease: a randomized double-blind placebo-controlled trial. Gastroenterology 120(6): 1330-8, 2001.

[81] Van Deventer SJ, Elson CO, and Fedorak RN. Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Crohn's Disease Study Group. Gastroenterology 113(2): 383-9, 1997.

[82] Sands BE, Bank S, Sninsky CA, Robinson M, Katz S, Singleton JW, Miner PB, Safdi MA, Galandiuk S, Hanauer SB, Varilek GW, Buchman AL, Rodgers VD, Salzberg B, Cai B, Loewy J, DeBruin MF, Rogge H, Shapiro M, and Schwertschlag US. Preliminary evaluation of safety and activity of recombinant human interleukin 11 in patients with active Crohn's disease. Gastroenterology 117(1): 58-64, 1999.

[83] Yacyshyn BR, Bowen-Yacyshyn MB, Jewell L, Tami JA, Bennett CF, Kisner DL, and Shanahan WR Jr. A placebo-controlled trial of ICAM-1 antisense oligonucleotide in the treatment of Crohn's disease. Gastroenterology 114(6): 1133-42, 1998.

[84] Wu S, and Craig R. Intense nutritional support in inflammatory bowel disease. Digestive Diseases and Sciences 40(4): 843-52, 1995.

[85] Greenberg GR, Fleming CR, Jeejeebhoy KN, Rosenberg IH, Sales D, and Tremaine WJ. Controlled trial of bowel rest and nutritional support in the management of Crohn's disease. Gut 29(10): 1309-15, 1988.

[86] Song HK, and Buzby GP. Nutritional support for Crohn's disease. Surgical Clinics of North America 81(1): 103-115, 2001.

[87] Ladefoged K, Hessov I, and Jarnum S. Nutrition in short-bowel syndrome. The Scandinavian Journal of Gastroenterology supplement 216: 122-31, 1996.

[88] Fernandez-Banares F, Cabre E, Gonzalez-Huix F, and Gassull MA. Enteral nutrition as primary therapy in Crohn's disease. Gut 35(1 supplement 1): S55-9, 1994.

[89] Geerling BJ, Stockbrugger RW, and Brummer RJ. Nutrition and inflammatory bowel disease: an update. The Scandinavian Journal of Gastroenterology supplement 230: 95-105, 1999.

[90] Giaffer MH, North G, and Holdsworth CD. Controlled trial of polymeric versus elemental diet in treatment of active Crohn's disease. The Lancet 335(8693): 816-9, 1990.

[91] Raouf AH, Hildrey V, Daniel J, Walker RJ, Krasner N, Elias E, and Rhodes JM. Enteral feeding as sole treatment for Crohn's disease: controlled trial of whole protein vs amino acid based feed and a case study of dietary challenge. Gut 32(6): 702-7, 1991.

[92] VanderAJ, Sherman JH, and Luciano DS. The digestion and absorption of food. In: VanderAJ, Sherman JH, and Luciano DS, editors. Human physiology: the mechanisms of bodily functions, 6th edition. McGraw Hill, pages 561-600, 1994.

[93] McGee DW, Beagley KW, Aicher WK, and McGhee JR. Transforming growth factor-beta enhanced interleukin-6 secretion by intestinal epithelial cells. Immunology 77(1): 7-12 , 1992.

[94] Dwinell MB, Eckmann L, Leopard JD, Varki NM, and Kagnoff MF. Chemokine receptor expression by human intestinal epithelial cells. Gastroenterology 117(2): 359-367, 1999.

[95] Bland PW, and Kambarage DM. Antigen handling by the epithelium and lamina propria macrophages. Gastroenterology Clinics of North America 20(3): 577-96 , 1991.

[96] Macfarlane GT, and Macfarlane S. Human colonic microbiota: Ecology, physiology and metabolic potential of intestinal bacteria. The Scandinavian Journal of Gastroenterology Supplement 222: 3-9, 1997.

[97] Pathmakanthan S, and Hawkey CJ. A lay doctor's guide to the inflammatory process in the gastrointestinal tract. Postgraduate Medicine Journal 76(900): 611-7,2000.

[98] Fuss Ij, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-γ, whereas ulcerativecolitis LP cells manifest increased secretion of IL-5. The Journal of Immunology 157(3): 1261-70, 1996.

[99] Elson C. The immunology of inflammatory bowel disease. In: Kirsner JB, editor. Inflammatory bowel disease, 5th edition. WB Saunders Company, pages 208-39, 2000.

[100] Trier JS. Structure and function of intestinal M cells. Gastroenterology Clinics of North America 20(3): 531-47, 1991.

[101] Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. Science 272(5258): 60-6 , 1996.

[102] Abbas AK, Lichtman AH, and Pober JS. Cytokines. In: Abbas AK, Lichtman AH, and Pober JS , editors. Cellular and Molecular Immunology, 4th edition. WB Saunders Company, pages 235-269, 2000.

[103] Akira S, Hirano T, Taga T, and Kishimoto T. Biology of multifunctional cytokines: IL-6 and related molecules. The FASEB Journal 4(11): 2860, 1990.

[104] Yang SK, Eckmann LG, Panja J, and Kagnoff MF. Differential and regulated expression of C-X-C, C-C and C-chemokines by human colon epithelial cells. Gastroenterology 113(4): 1214-23, 1997.

[105] Podolsky D, and Fiocchi C. Cytokines, chemokines, growth factors, eicosanoids, and other bioactive molecules in inflammatory bowel disease. In: Kirsner JB (ed). Inflammatory bowel disease, 5th edition. WB Saunders Company, pp. 191-207, 2000.

[106] Jobin C, Haskill S, Mayer L, Panja A, and Sartor RB. Evidence for an altered regulation of IκBα degradation in human colonic epithelial cells. The Journal of Immunology 158(1): 226-34, 1997.

[107] Thanos D and Maniatis T. NF-κB: a lesson in family values. Cell 80(4): 529-32, 1995.

[108] Finco TS, and Baldwin AS. Mechanistic aspects of NF-κB regulation: the emerging role of phosphorylation and proteolysis. Immunity 3(3): 263-72, 1995.

[109] Arenzana-Seisdedos F, Turpin P, Rodriguez M, Thomas D, Hay T, Virelizier JL, and Dargemont C. Nuclear localization of IκBα promotes active transport of NF-κB from the nucleus to the cytoplasm. The Journal of Cell Science 110(pt 3): 369-78, 1997.

[110] Barnes PJ, and Karin M. Nuclear factor-B, a pivotal transcription factor in chronic inflammatory diseases. The New England Journal of Medicine 336(15): 1066-71, 1997.

[111] Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. Oncogene 18(49): 6853-66, 1999.

[112] Jobin C, and Sartor RB. The IκB/NF-κB system: a key determinant of mucosal inflammation and protection. The American Journal of Physiology, Cell Physiology 278(3): C451-462, 2000.

[113] Tak PP, and Firestein GS. NF-κB: a key role in inflammatory diseases. The Journal of Clinical Investigation 107(1): 7-11, 2001.

[114] Podolsky DK. Inflammatory Bowel Disease. The New England Journal of Medicine 325(13): 928-37, 1991.

[115] Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362(6423): 801-9, 1993.

[116] Mattson MP, and Camandola S. NF-κB in neuronal plasticity and neurodegenerative disorders. The Journal of Clinical Investigation 107(3): 247-54, 2001.

[117] Barkett M, and Gilmore TD. Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene 18(49): 6910-24, 1999.

[118] Chen F, Castranova V, Shi C, and Demers L. New insights into the role of nuclear factor-κB a ubiquitous transcription factor in the initiation of disease. Clinical Chemistry 45(1): 7-17, 1999.

[119] Seitz CS, Lin Q, Deng H, and Khavari PA. Alterations in NF-κB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-κB. Proceedings of the National Academy of Science 95(5): 2307-12, 1998.

[120] Guttridge DC, Albanese C, Reuther JY, Pestell RG, and Baldwin AS. NF-κB controls cell growth and differentiation through transcriptional regulation of cylcin D1. Molecular and Cellular Biology 19(8): 5785-99, 1999.

[121] Rayet B, and Gelinas C. Aberrant rel/NF-κB genes and activity in human cancer. Oncogene 18(49): 6938-47, 1999

[122] Dejardin E, Bonizzi G, Bellahcene A, Castrovono V, Merville MP, and Bours V. Highly expressed p100/p52 (NF-κB2) sequesters other NF kappa-B related proteins in the cytoplasm of human breast cancer cells. Oncogene 11(9): 1835-41, 1995.

[123] Finco TS, Westwick JK, Norris JL, Beg AA, Der CJ, and Bladwin AS. Oncogenic Ha-Ras-induced signaling activates NF-κB transcriptional activity, which is required for cellular transformation. The Journal of Biological Chemistry 272(39): 24113-6, 1997.

[124] Reuther JY, Reuther GW, Cortez D, Pendergast M and Baldwin AS. A requirement for NF-κB activation in Bcr-Abl-mediated transformation. Genes and Development 12(7): 968-81, 1998.

[125] Olaison G, Sjodahl R, and Tagesson C. Abnormal intestinal permeability in Crohn's disease. A possible pathogenic factor. The Scandinavian Journal of Gastroenterology 25(4): 321-8, 1990.

[126] Hollander D. The intestinal permeability barrier. A hypothesis to its regulation and involvement in Crohn's disease. The Scandinavian Journal of Gastroenterology 27(9): 721-6, 1992.

[127] Gassler N, Rohr C, Schneider A, Kartenbeck J, Bach A, Obermuller N, Otto HT, and Austbach F. Inflammatory bowel disease is associated with changes of enterocytic junctions. The American Journal of Gastroenterology and Liver Physiology 281(1): G216-28, 2001.

[128] Soderholm JD, Peterson KH, Olaison G, Franzen LE, Westrom B, Magnusson KE, and Sjodahl R. Epithelial permeability to proteins in the noninflamed ileum of Crohn's disease? Gastroenterology 117(1): 65-72, 1999.

[129] Marin ML, Greenstein AJ, Geller SA, Gordon RE, and Aufses AH. A freeze fracture study of Crohn's disease of the terminal ileum: changes in epithelial tight junction organization. The American Journal of Gastroenterology 78(9): 537-47, 1983.

[130] Peeters M, Ghoos Y, Maes B, Hiele M, Geboes K, Ventrapen G, and Rutgeerts P. Increased permeability of macroscopically normal small bowel in Crohn's disease. Digestive Disease and Science 39(10): 2170-6, 1994.

[131] Hilsden RJ, Meddings JB, and Sutherland LR. Intestinal permeability changes in response to acetylsalicylic acid in relatives of patients with Crohn's disease. Gastroenterology 110(5): 1395-1403, 1996.

[132] Munkholm P, Langhloz E, Hollander D, Thornberg K, Orholm M, Katz KD, and Binder V. Intestinal permeability in patients with Crohn's disease and ulcerative colitis and their first degree relatives. Gut 35(1): 68-72, 1994.

[133] Malin M, Isolauri E, Pikkarainen P, Karikoski R, and Isolauri J. Enhanced absorption of macromolecules. A secondary factor in Crohn's disease. Digestive Disease and Science 41(7): 1423-8, 1996.

[134] Colgan SP, Parkos CA, Delp C, Arnaout MA, and Madara JL. Neutrophil migration across cultured intestinal epithelial monolayers is modulated by epithelial exposure to IFN-γ in a highly polarized fashion. The Journal of Cell Biology 120(3): 785-98, 1993.

[135] Colgan SP, Resnick MB, Parkos CA, Delp-Archer C, McGuirk D, Baccara AE, Weller PF, and Madara JL. IL-4 directly modulates function of a model human intestinal epithelium. The Journal of Immunology, 153(5): 2122-9, 1994.

[136] Schmitz H, Fromm M, Bentzel CJ, Scholz P, Detjen K, Mankhertz J, Bode H, Epple HJ, Riecken EO, and Schulzke JD. Tumor necrosis factor-α (TNF-α) regulates the epithelial barrier in the human intestinal cell line HT-29/B6. The Journal of Cell Science 112(Pt 1): 137-46, 1999.

[137] Fiocchi C, Hilfiker ML, Youngman KR, Doerder NC, and Finke JH. Interleukin 2 activity of human intestinal mucosa mononuclear cells. Decreased levels in inflammatory bowel disease. Gastroenterology 86(4): 734-42, 1984.

[138] Kusugami K, Youngman KR, West GA, and Fiocchi C. Intestinal immune reactivity to interleukin 2 differs among Crohn's disease, ulcerative colitis, and controls. Gastroenterology 97(1): 1-9, 1989.

[139] Fais S, Capobianchi MR, Pallone F, Di Marco P, Boirivant M, Dianzani F, and Torsoli A. Spontaneous release of interferon gamma by intestinal lamina propria lymphocytes in Crohn's disease. Kinetics of in vitro response to interferon gamma inducers. Gut 32(4): 403-7, 1991.

[140] Fais S, Capobianchi MR, Silvestri M, Mercuri F, Pallone F, and Dianzani F. Interferon expression in Crohn's disease patients: increased interferon-gamma and -alpha mRNA in the intestinal lamina propria mononuclear cells. The Journal of Interferon Research 14(5): 235-8, 1994.

[141] Breese E, Braegger CP, Corrigan CJ, Walker-Smith JA, and MacDonald TT. Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. Immunology 78(1): 127-31, 1993.

[142] Fuss IJ, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, and Strober W. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. The Journal of Immunology 157(3): 1261-70, 1996.

[143] Matsuura T, West GA, Klein JS, and . Immune activation gene products are resistant to IL-4 inhibitory activity in Crohn's disease. Gastroenterology 104: A739, 1993.

[144] Desreumaux P, Brandt E, Gambiez L, Emilie D, Geboes K, Klein O, Ectors N, Cortot A, Capron M, and Colombel JF. Distinct cytokine patterns in early and chronic ileal lesions of Crohn's disease. Gastroenterology 113(1): 118-26, 1997.

[145] Izzo RS, Witkon K, Chen AI, Hadjiyane C, Weinstein MI, and Pellecchia C. The Scandinavian Journal of Gastroenterology 28(4): 296-300, 1993.

[146] Gibson P, and Rosella O. Interleukin 8 secretion by colonic crypt cells in vitro: response to injury suppressed by butyrate and enhanced in inflammatory bowel disease. Gut 37(4): 536-43, 1995.

[147] Z'Graggen K, Walz A, Mazzucchelli L, Strieter RM, and Mueller C. The C-X-C chemokine ENA-78 is preferentially expressed in intestinal epithelium in inflammatory bowel disease. Gastroenterology 113(3): 808-16, 1997.

[148] Reinecker HC, Loh EY, Ringler DJ, Mehta A, Rombeau JL, and MacDermott RP. Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. Gastroenterology 108(1): 40-50, 1995.

[149] Grimm MC, and Doe WF. Chemokines in inflammatory bowel disease mucosa: expression of RANTES, macrophage inflammatory protein (MIP)-1α, MIP-1β, and γ-interferon-inducible protein 10 by macrophages,

lymphocytes, endothelial cells, and granulomas. Inflammatory Bowel Disease 2: 88, 1996.

[150] Dionne S, Hiscott J, D'Agata I, Duhaime A, and Seidman EG. Quantitative PCR analysis of TNF-alpha and IL-1 beta mRNA levels in pediatric IBD mucosal biopsies. Digestive Diseases and Science 42(7): 1557-66, 1997.

[151] Murch SH, Lamkin VA, Savage MO, Walker-Smith JA, and MacDonald TT. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. Gut 32(8): 913-7, 1991.

[152] Mahida TR, Kurlak L, Gallagher A, and Hawkey CJ. High circulating concentrations of interleukin-6 in active Crohn's disease but not ulcerative colitis. Gut 32(12): 1531-4, 1991.

[153] Kucharzik T, Stoll R, Lugering N, and Domschke W. Circulating anti-inflammatory cytokine IL-10 in patients with inflammatory bowel disease (IBD). Clinical and Experimental Immunology 100(3): 452-6, 1995.

[154] Babyatsky MW, Rossiter G, and Podolsky DK. Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. Gastroenterology 110(4): 975-84, 1998.

[155] Andus T, Daig R, Vogl D, Aschenbrenner E, Lock G, Hollerbach S, Kollinger M, Scholmerich J, Gross V. Imbalance of the interleukin 1 system in colonic mucosa--association with intestinal inflammation and interleukin 1 receptor antagonist genotype 2. Gut 41(5): 651-7, 1997.

[156] Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, Knuechel R, Baeuerle PA, Scholmerich J, and Gross V. Nuclear factor kappa B is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 115(2): 357-69, 1998.

[157] Hellerbrand C, Jobin C, Sartor RB Brener DA. NF-κB mediates cytokine induced expression of ICAM-1 in IEC-6 cells. Gastroenterology 110: A 329, 1996.

[158] Neurath MF, Pettersson S, Meyer zum Buschenfelde KH, and Strober W. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. Nature Medicine 2(9): 998-1004, 1996.

[159] Muller JM, Ziegler-Heitbrock HW, and Baeuerle PA. Nuclear factor kappa B, a mediator of lipopolysaccharide effects. Immunobiology 187(3-5): 233-56. 1993.

[160] Baeuerle PA, and Henkel T. Function and activation of NF-kappa B in the immune system. The Annual Reviews in Immunology 12: 141-79, 1994.

[161] Gross V, Andus T, Daig R, Aschenbrenner E, Scholmerich J, and Falk W. Regulation of interleukin-8 production in a human colon epithelial cell line (HT-29). Gastroenterology 108(3): 653-61, 1995.

[162] Kusugami K, Fukatsu A, Tanimoto M, Shinoda M, Haruta J, Kuroiwa A, Ina K, Kanayama K, Ando T, Matsuura T, et al. Elevation of interleukin-6

in inflammatory bowel disease is macrophage- and epithelial cell-dependent. Digestive Diseases and Science 40(5): 949-59, 1995.

[163] Iademarco MF, McQuillan JJ, Rosen GD, and Dean DC. Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). The Journal of Biological Chemistry 267(23): 16323-9, 1992.

[164] Elewaut D, DiDonato JA, Kim JM, Truong F, Eckmann L, and Kagnoff MF. NF-kappa B is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. The Journal of Immunology 163(3): 1457-66, 1999.

[165] Neurath MF, Fuss I, Schurmann G, Pettersson S, Arnold K, Muller-Lobeck H, Strober W, Herfarth C, and Buschenfelde KH. Cytokine gene transcription by NF-kappa B family members in patients with inflammatory bowel disease. Annals of the New York Academy of Science 859: 149-59, 1998.

[166] Kehrer JP. Free radicals as mediators of tissue injury and disease. Critical Reviews in Toxicology 23(1): 21-48, 1993.

[167] Kehrer JP, and Smith C. Free radicals in biology: sources, reactivities and role in the etiology of human diseases. In: Balz Frei, editor. Natural antioxidants in human health and disease. Academic Press, pp. 25-63, 1994.

[168] Pryor WA. Oxy-radicals and related species: their formation, lifetimes, and reactions. Annual Review of Physiology 48: 657-67, 1986.

[169] Hurst JK, and Barrette WC. Leukocytic oxygen activation and microbicidal oxidative toxins. Critical Reviews in Biochemistry and Molecular Biology 24(4): 271-328, 1989.

[170] Boveris A, and Cadenas E. Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. FEBS Letters 54(3): 311-4, 1975.

[171] Haliwell B. Free radicals and antioxidants: a personal view. Nutrition Reviews 52(8): 253-65, 1994.

[172] Fridovich I. Superoxide dismutases. Advances in Enzymology and Related Areas of Molecular Biology 58: 61-97, 1986.

[173] Haber F, and Weiss JJ. The catalytic decomposition of H_2O_2 by iron salts. Proceedings of the Royal Society of London (Series A) 147: 332-51, 1934.

[174] Gordeuk VR, McLaren GD, and Samowitz W. Etiologies, consequences and treatment of iron overload. Critical Reviews in Clinical Laboratory Sciences 31(2): 89-133, 1994.

[175] Bacon BR, and Britton RS. The pathology of hepatic iron overload: a free radical-mediated process? Hepatology 11(1): 127-37, 1990.

[176] Ames BN, Shigenaga MK, and Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences of the USA 90(17): 7915-22, 1993.

[177] Davies KJ. Intracellular proteolytic systems may function as secondary antioxidant defenses: an hypothesis. The Journal of Free Radical Biology and Medicine 2(3): 155-73, 1986.

[178] Stadtman ER. Protein oxidation and aging. Science 257(5074): 1220-4, 1992.

[179] Halliwell B, and Aruoma OI. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Letters 281(1-2): 9-19, 1991.

[180] Christen Y. Oxidative stress and Alzheimer disease. The American Journal of Clinical Nutrition 71(2): 621S-9S, 2000.

[181] Cochrane CG. Cellular injury by oxidants. The American Journal of Medicine 91(3C): 23S-30S, 1991.

[182] Halliwell B, and Chirico S. Lipid peroxidation: its mechanism, measurement, and significance. The American Journal of Clinical Nutrition 57(5 suppl): 715S-25S, 1993.

[183] Chance B, Sies H, and Boveris A. Hydroperoxide metabolism in mammalian organs. Physiology Reviews 59(3): 527-605, 1979.

[184] Briviba K and Sies H. Nonenzymatic antioxidant defense systems. In: Balz Frei, editor. Natural antioxidants in human health and disease. Academic press, pp. 107-128, 1994.

[185] Lu SC. Regulation of hepatic glutathione synthesis: current concepts and controversies. The FASEB Journal 13(10): 1169-83, 1999.

[186] Wefers H, and Sies H. The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. The European Journal of Biochemistry 174(2): 353-7, 1988.

[187] Di Mascio P, Murphy ME, and Sies H. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. The American Journal of Clinical Nutrition 53(1 Suppl): 194S-200S, 1991.

[188] Bowie A, and O'Neill LA. Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. Biochemical Pharmacology 59(1): 13-23, 2000.

[189] Sen CK and Packer L. Antioxidant and redox regulation of gene transcription. FASEB Journal 10(7): 709-20, 1996.

[190] Schreck R, Rieber P, and Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. EMBO Journal 10(8): 2247-58, 1991.

[191] Manna SK, Zhang HJ, Yan T, Oberley LW, and Aggarwal BB. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. The Journal of Biological Chemistry 273(21): 13245-54, 1998.

[192] Wang X, Martindale JL, Liu Y, and Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. The Biochemical Journal 333 (Pt 2): 291-300, 1998.

[193] Meier B, Radeke HH, Selle S, Younes M, Sies H, Resch K, and Habermehl GG. Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-alpha. The Biochemical Journal 263(2): 539-45, 1989.

[194] Bonizzi G, Piette J, Schoonbroodt S, Greimers R, Havard L, Merville MP, and Bours V. Reactive oxygen intermediate-dependent NF-kappaB activation by interleukin-1beta requires 5-lipoxygenase or NADPH oxidase activity. Molecular and Cellular Biology 19(3): 1950-60, 1999.

[195] Bonizzi G, Dejardin E, Piret B, Piette J, Merville MP, and Bours V. Interleukin-1 beta induces nuclear factor kappa B in epithelial cells independently of the production of reactive oxygen intermediates. The European Journal of Biochemistry 242(3): 544-9, 1996.

[196] Schreck R, Meier B, Mannel DN, Droge W, and Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. The Journal of Experimental Medicine 175(5): 1181-94, 1992.

[197] Schmidt KN, Amstad P, Cerutti P, and Baeuerle PA. The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-kappa B. Chemistry and Biology 2(1): 13-22, 1995.

[198] Li N, and Karin M. Is NF-kappaB the sensor of oxidative stress? FASEB Journal 13(10): 1137-43, 1999.

[199] Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, and Packer L. Redox regulation of NF-kappa B activation. Free Radical Biology and Medicine 22(6): 1115-26, 1997.

[200] Anderson MT, Staal FJ, Gitler C, Herzenberg LA, and Herzenberg LA. Separation of oxidant-initiated and redox-regulated steps in the NF-kappa B signal transduction pathway. Proceedings of the National Academy of Science of the U S A 91(24): 11527-31, 1994.

[201] Natarajan R, Ghosh S, Fisher BJ, Diegelmann RF, Willey A, Walsh S, Graham MF, and Fowler AA 3rd. Redox imbalance in Crohn's disease intestinal smooth muscle cells causes NF-kappaB-mediated spontaneous interleukin-8 secretion. The Journal of Interferon and Cytokine Research 21(6): 349-59, 2001.

[202] Keshavarzian A, Sedghi S, Kanofsky J, List T, Robinson C, Ibrahim C, and Winship D. Excessive production of reactive oxygen metabolites by inflamed colon: analysis by chemiluminescence probe. Gastroenterology 103(1): 177-185, 1992.

[203] Simmonds NJ, Allen RE, Stevens TR, Van Someren RN, Blake DR, and Rampton DS. Chemiluminescence assay of mucosal reactive oxygen

metabolites in inflammatory bowel disease. Gastroenterology 103(1): 186-96, 1992.

[204] D'Odorico A, D'Inca R, Mestriner C, Di Leo V, Ferronato A, and Sturniolo GC. Influence of disease site and activity on peripheral neutrophil function in inflammatory bowel disease. Digestive Diseases and Science 45(8): 1594-600, 2000.

[205] Curran FT, Allan RN, and Keighley MR. Superoxide production by Crohn's disease neutrophils. Gut 32(4): 399-402, 1991.

[206] Verspaget HW, Pena AS, Weterman IT, and Lamers CB. Diminished neutrophil function in Crohn's disease and ulcerative colitis identified by decreased oxidative metabolism and low superoxide dismutase content. Gut 29(2): 223-8, 1988.

[207] Williams JG, Hughes LE, and Hallett MB. Toxic oxygen metabolite production by circulating phagocytic cells in inflammatory bowel disease. Gut 31(2): 187-93, 1990.

[208] Kitahora T, Suzuki K, Asakura H, Yoshida T, Suematsu M, Watanabe M, Aiso S, and Tsuchiya M. Active oxygen species generated by monocytes and polymorphonuclear cells in Crohn's disease. Digestive Diseases and Sciences 33(8): 951-5, 1988.

[209] Wendland BE, Aghdassi E, Tam C, Carrrier J, Steinhart AH, Wolman SL, Baron D, and Allard JP. Lipid peroxidation and plasma antioxidant micronutrients in Crohn disease. American Journal of Clinical Nutrition 74(2): 259-64, 2001.

[210] Mingrone G, Greco AV, Benedetti G, Capristo E, Semeraro R, Zoli G, and Gasbarrini G. Increased resting lipid oxidation in Crohn's disease. Digestive Diseases and Sciences 41(1): 72-6, 1996.

[211] Ahnfelt-Ronne I, Nielsen OH, Christensen A, Langholz E, Binder V, and Riis P. Clinical evidence supporting the radical scavenger mechanism of 5-aminosalicylic acid. Gastroenterology 98(5 Pt 1): 1162-9, 1990.

[212] Levy E, Rizwan Y, Thibault L, Lepage G, Brunet S, Bouthillier L, and Seidman E. Altered lipid profile, lipoprotein composition, and oxidant and antioxidant status in pediatric Crohn disease. The American Journal of Clinical Nutrition 71(3): 807-15, 2000.

[213] Lih-Brody L, Powell SR, Collier KP, Reddy GM, Cerchia R, Kahn E, Weissman GS, Katz S, Floyd RA, McKinley MJ, Fisher SE, and Mullin GE. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. Digestive Diseases and Sciences 41(10): 2078-86, 1996.

[214] McKenzie SJ, Baker MS, Buffinton GD, and Doe WF. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. The Journal of Clinical Investigation 98(1): 136-41, 1996.

[215] D'Odorico A, Bortolan S, Cardin R, D'Inca' R, Martines D, Ferronato A, and Sturniolo GC. Reduced plasma antioxidant concentrations and increased oxidative DNA damage in inflammatory bowel disease. The Scandinavian Journal of Gastroenterology 36(12): 1289-94, 2001.

[216] Kuchino Y, Mori F, Kasai H, Inoue H, Iwai S, Miura K, Ohtsuka E, and Nishimura S. Misreading of DNA templates containing 8-hydroxydeoxyguanosine at the modified base and at adjacent residues. Nature 327(6117): 77-9, 1987.

[217] Markowitz MM, Rozen P, Pero RW, Tobi M, and Miller DG. Hydrogen peroxide induced adenosine diphosphate ribosyl transferase (ADPRT) response in patients with inflammatory bowel disease. Gut 29(12): 1680-6, 1988.

[218] Grisham MB, MacDermott RP, and Deitch EA. Oxidant defense mechanisms in the human colon. Inflammation 14(6): 669-80, 1990.

[219] Mulder TPJ, Verspaget HW, Janssens AR, de Bruin PAF, Pena AS, and Lamers CBHW. Decrease in two intestinal copper/zinc containing proteins with antioxidant function in inflammatory bowel disease. Gut 32(10): 1146-50, 1991.

[220] Hoffenberg EJ, Deutsch J, Smith S, and Sokol RJ. Circulating antioxidant concentrations in children with inflammatory bowel disease. The American Journal of Clinical Nutrition 65(5): 1482-8, 1997.

[221] Geerling BJ, Badart-Smook A, Stockbrugger RW, and Brummer RJ. Comprehensive nutritional status in patients with long-standing Crohn disease currently in remission. The American Journal of Clinical Nutrition 67(5): 919-26, 1998.

[222] Geerling BJ, v Houwelingen AC, Badart-Smook A, Stockbrugger RW, and Brummer RJ. The relation between antioxidant status and alterations in fatty acid profile in patients with Crohn disease and controls. The Scandinavian Journal of Gastroenterology 34(11): 1108-16, 1999.

[223] Rannem T, Ladefoged K, Hylander E, Hegnhoj J, and Jarnum S. Selenium status in patients with Crohn's disease. The American Journal of Clinical Nutrition 56(5): 933-7, 1992.

[224] Sido B, Hack V, Hochlehnert A, Lipps H, Herfarth C, and Droge W. Impairment of intestinal glutathione synthesis in patients with inflammatory bowel disease. Gut 42(4): 485-92, 1998.

[225] Ringstad J, Kildebo S, and Thomassen Y. Serum selenium, copper, and zinc concentrations in Crohn's disease and ulcerative colitis. The Scandinavian Journal of Gastroenterology 28(7): 605-8, 1993.

[226] Kuroki F, Iida M, Tominaga M, Matsumoto T, Hirakawa K, Sugiyama S, and Fujishima M. multiple vitamin status in Crohn's disease: correlation with disease activity. Digestive Diseases and Sciences 38(9): 1614-8, 1993.

[227] Buffinton GD, and Doe W. Depleted mucosal antioxidant defences in inflammatory bowel disease. Free Radical Biology and Medicine 19(6): 911-8, 1995.

[228] Blazovics A, Kovacs A, Lugasi A, Hagymasi K, Biro L, and Feher J. Antioxidant defense in erythrocytes and plasma of patients with active and quiescent Crohn disease and ulcerative colitis: a chemiluminescent study. Clinical Chemistry 45(6 Pt 1): 895-6, 1999.

[229] Fretland DJ, Widowski Dl, Anglin CP, Wlash RE, Levin S, Riley D, Weiss RH, and Gaginella TS. Superoxide dismutase modulates acetic acid-induced colitis in rodents. Gastroenterology 100: A581, 1991.

[230] Yoshikawa T, Takahashi S, and Kondo M. Possible role of free radicals in the chronic inflammation of the gut. EXS 62: 353-8, 1992.

[231] Moyana T, and Lalonde JM. Carrageenan-induced intestinal injury: possible role of oxygen free radicals. Annals of Clinical and Laboratory Science 21(4): 258-63, 1991.

[232] Keshavarzian A, Haydek T, Zabihi R, Doria M, D'Astice M, and Sorenson JR. Agents capable of eliminating reactive oxygen species. Catalase, WR-2721, or Cu(II)2(3,5-DIPS)4 decrease experimental colitis. Digestive Diseases and Sciences 37(12): 1866-73, 1992.

[233] Lash L, Hagen TM, and Jones DP. Exogenous glutathione protects intestinal epithelial cells from oxidative injury. Proceedings of the National Academy of Sciences USA 83(13): 4641-5, 1986.

[234] Von Ritter C, Grisham MB, Hollwarth M, Inauen W, and Granger DN. Neutrophil-derived oxidants mediate formyl-methionyl-leucyl-phenylalanine-induced increases in mucosal permeability in rats. Gastroenterology. 97(3): 778-80, 1989.

[235] Reimund JM, Allison AC, Muller CD, Dumont S, Kenney JS, Baumann R, Duclos B, and Poindron P. Antioxidants inhibit the in vitro production of inflammatory cytokines in Crohn's disease and ulcerative colitis. The European Journal of Clinical Investigation 28(2): 145-50, 1998.

[236] Emerit J, Pelletier S, Tosoni-Verlignue D, and Mollet M. Phase II trial of copper zinc superoxide dismutase (CuZnSOD) in treatment of Crohn's disease. Free Radical Biology and Medicine 7(2): 145-9, 1989.

[237] Gaginella TS, and Walsh RE. Sulfasalazine. Multiplicity of action. Digestive Diseases and Sciences 37(6): 801-12, 1992.

[238] Craven PA, Pfanstiel J, Saito R, and DeRubertis FR. Actions of sulfasalazine and 5-aminosalicylic acid as reactive oxygen scavengers in the suppression of bile acid-induced increases in colonic epithelial cell loss and proliferative activity. Gastroenterology 92(6): 1998-2008, 1987.

[239] Von Ritter C, Grisham B, and Granger DN. Sulfasalazine metabolites and dapsone attenuate formyl-methionyl-leucyl-phenylalanine-induced mucosal injury in rat ileum. Gastroenterology 96(3): 811-6, 1989.

[240] Yamada T, Volkmer C, and Grisham MB. Antioxidant properties of ASA: potential mechanism for its anti-inflammatory activity. The Canadian Journal of Gastroenterology 4: 295-302, 1990.

[241] Grisham MB. Oxidants and free radicals in IBD. The Lancet 344(8926): 859-61, 1994.

[242] Kuppusamy P and Zweier HL. Characterization of free radical generation by xanthine oxidase. Evidence for hydroxyl radical generation. The Journal of Biological Chemistry 264(17): 9880-4, 1989.

[243] Halliwell B and Gutteridge JM. Iron and free radicals reaction: two aspects of antioxidant protection. TIBS 11: 372-5, 1986.

[244] Grisham MB and Granger DN. Neutrophil-mediated mucosal injury. Role of reactive oxygen metabolites. Digestive Diseases and Sciences 33(3): 6S-15S, 1988.

[245] Gross V, Arndt H, Andus T, Palitzsch KD and Schlomerich J. Free radicals in inflammatory bowel diseases pathophysiology and therapeutic implications. Hepatogastroenterology 41(4): 320-7, 1994.

[246] Dionne S, Ruemmele FM and Seidman EG. Immunopathogenesis of inflammatory bowel disease: role of cytokines and immune cell-enterocyte interactions. Nestlé Nutrition Workshop Series, vol 2. Karker Switzerland, pp 41-57, 1999.

[247] DeWitt RC and Kudsk KA. The gut's role in metabolism, mucosal barrier function and gut immunology. Infectious Disease Clinics of North America 13(2): 465-81, 1999.

[248] Levy E, Mehran M and Seidman E. Caco-2 cells as a model for intestinal lipoprotein synthesis and secretion. The FASEB Journal 9(8): 626-35, 1995.

[249] Mehran M, Levy E, Bendayan M and Seidman E. Lipid, apolipoprotein, and lipoprotein synthesis and secretion during cellular differentiation in Caco-2 cells. In Vitro Cell Dev Biol Anim 33(2): 118-28, 1997.

[250] Baker SS and Baker RD. Antioxidant enzymes in the differentiated Caco-2 cell line. In Vitro Cell Dev Biol 28A(9-10): 643-7, 1992.

[251] Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N et al. Enterocye-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biology of the Cell 47: 323-33, 1983.

[252] Babbs CF. Oxygen radicals in ulcerative colitis. Free Radical Biology and Medicine 13(2): 169-81, 1992.

[253] Welch KD, Davis TZ, Van Eden ME and Aust SD. Deleterious iron-mediated oxidation of biomolecules. Free Radical Biology and Medicine 32(7): 577-83, 2002.

[254] Jouzeau J-Y, Terlain B, Abid A, Nedelec E and Netter P. Cyclo-oxygenase isoenzymes. How recent findings affect thinking about nonsteroidal anti-inflammatory drugs. Drugs 53(4): 563-82, 1997.

[255] Scheiman JM. NSAIDs, gastrointestinal injury, and cytoprotection. Gastroenterology Clinics of North America 25(2): 279-98, 1996.

[256] Hershman HR. Prostaglandin synthase 2. Biochimica et Biophysica Acta 1299: 125-140, 1996.

[257] Jobin C, Marteau O, Han DS, and Balfour Sartor R. Specific NF-kappaB blockade selectively inhibits tumour necrosis factor-alpha-induced COX-2 but not constitutive COX-1 gene expression in HT-29 cells. Immunology 95(4): 537-43, 1998.

[258] Schmedtje JF, Ji YS, Liu WL, DuBois RN, and Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. The Journal of Biological Chemistry 272(1): 601-8, 1997.

[259] Poligone B and Baldwin AS. Positive and negative regulation of NF-kappaB by COX-2: roles of different prostaglandins. The Journal of Biological Chemistry 276(42): 38658-64, 2001.

[260] Kumagai T, Kawamoto Y, Nakamura Y, Hatayama I, Satoh K, Osawa T, and Uchida K. 4-hydroxy-2-nonenal, the end product of lipid peroxidation, is a specific inducer of cyclooxygenase-2 gene expression. Biochemical and Biophysical Research Communications 273(2): 437-41, 2000.

[261] Feng L, Xia Y, Garcia GE, Hwang D, and Wilson CB. Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha, and lipopolysaccharide. The Journal of Clinical Investigation 95(4): 1669-75, 1995.

[262] Singer II, Kawka DW, Schloemann S, Tessner T, Riehl T, and Stenson WF. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. Gastroenterology 15(2): 97-306, 1998.

[263] Muller-Decker K, Albert C, Lukanov T, Winde G, Marks F, and Furstenberger G. Cellular localization of cyclo-oxygenase isozymes in Crohn's disease and colorectal cancer. International Journal of Colorectal Disease 14(4-5): 212-8, 1999.

[264] Springer TA, Dustin ML, Kishimoto TK, and Marlin SD. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. Annual Review of Immunology 5: 223-52, 1987.

[265] Albelda SM, Smith CW, and Ward PA. Adhesion molecules and inflammatory injury. The FASEB Journal 8(8): 504-12, 1994.

[266] Elgavish A. Effects of Escherichia coli and E. coli lipopolysaccharides on the function of human ureteral epithelial cells cultured in serum-free medium. Infection and Immunity 61(8): 3304-12, 1993.

[267] Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, and Sartor RB. Curcumin blocks cytokine-mediated NF-kappa B activation and

proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. The Journal of Immunology 163(6): 3474-83, 1999.

[268] Lakshminarayanan V, Beno DWA, Costa RH, and Roebuck KA. Differential regulation of interleukin-8 and intercellular adhesion molecule-1 by H2O2 and tumor necrosis factor-alpha in endothelial and epithelial cells. The Journal of Biological Chemistry 272(52): 32910-8, 1997.

[269] Vainer B, Nielsen OH, and Horn T. Comparative studies of the colonic in situ expression of intercellular adhesion molecules (ICAM-1, -2, and -3), beta2 integrins (LFA-1, Mac-1, and p150,95), and PECAM-1 in ulcerative colitis and Crohn's disease. The American Journal of Surgical Pathology 24(8): 1115-24, 2000.

[270] Streiter RM, Kunkel SL, Showell HJ, Remick DG, Phan SH, Ward PA, and Marks RM. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1 beta. Science 243 (4897): 1467-9, 1989.

[271] Nakamura H, Yoshimura K, Jaffe HA, and Crystal RG. Interleukin-8 gene expression in human bronchial epithelial cells. The Journal of Biological Chemistry 266(29): 19611-7, 1991.

[272] Mukaida N, Mahe Y, and Matsushima K. Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. The Journal of Biological Chemistry 265(34): 21128-33, 1990.

[273] Yasumoto K, Okamoto S, Mukaida N, Murakami S, Mai M, and Matsushima K. Tumor necrosis factor alpha and interferon gamma synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-κB-like binding sites of the interleukin 8 gene. The Journal of Biological Chemistry 267(31): 22506-11, 1992.

[274] DeForge LE, Preston AM, Takeuchi E, Kenney J, Boxer LA, and Remick DG. Regulation of interleukin 8 gene expression by oxidant stress. The Journal of Biological Chemistry 268(34): 25568-76, 1993.

[275] DeForge LE, Fantone JC, Kenney JS, and Remick DG. Oxygen radical scavengers selectively inhibit interleukin 8 production in human whole blood. The Journal of Clinical Investigation 90(5): 2123-9, 1992.

[276] Mazzuchelli L, Hauser C, Z'Graggen K, Wagner J, Hess M, Laissue JA, and Mueller C. Expression of interleukin-8 gene in inflammatory bowel disease is related to the histological grade of active inflammation. The American Journal of Pathology 144(5): 997-1007, 1994.

[277] Grimm MC, Elsbury SK, Pavli P, and Doe WF. Interleukin 8: cells of origin in inflammatory bowel disease. Gut 38(1): 90-8, 1996.

[278] Nielsen OH, Rudiger N, Gaustadnes M, and Horn T. Intestinal interleukin-8 concentration and gene expression in inflammatory bowel disease. The Scandinavian Journal of Gastroenterology 32(10): 1028-34, 1997.

[279] Meyer M, Schreck R, and Baeuerle PA. H2O2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO Journal 12(5): 2005-15, 1993.

[280] Pinkus R, Weiner LM, and Daniel V. Role of oxidants and antioxidants in the induction of AP-1, NF-kappaB, and glutathione S-transferase gene expression. The Journal of Biological Chemistry 271(23): 13422-9, 1996.

[281] Shrivastava A and Aggarwal BB. Antioxidants differentially regulate activation of nuclear factor-kappa B, activator protein-1, c-jun amino-terminal kinases, and apoptosis induced by tumor necrosis factor: evidence that JNK and NF-kappa B activation are not linked to apoptosis. Antioxidants and Redox Signalling 1(2): 181-91, 1999.