Chapter 3

Expression of Nitric Oxide Synthases, and Formation of Nitrotyrosine and Reactive Oxygen Species in Inflammatory Bowel Disease

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ABSTRACT

Nitric oxide (NO) and reactive oxygen species (ROS) are important mediators in the pathogenesis of inflammatory bowel disease (IBD). NO in IBD can either be harmful or protective. NO can react with superoxide anions (O$_2^-$) yielding the toxic oxidizing agent peroxynitrite (ONOO$^-$. Peroxynitrile induces nitration of tyrosine residues (nitrotyrosine) leading to changes of protein structure and function. The aim of this study was to identify the cellular source of inducible nitric oxide synthase (iNOS), and to localise superoxide anion producing cells in mucosal biopsies from patients with active IBD. Additional studies were performed to look at nitrotyrosine formation as a measure of peroxynitrite mediated tissue damage. For this antibodies against iNOS, eNOS, and nitrotyrosine were used. ROS producing cells were detected cytochemically. Inflamed mucosa of patients with active IBD showed intense iNOS staining in the epithelial cells. iNOS could not be detected in non-inflamed mucosa of IBD patients and control subjects. eNOS was present in blood vessels, without any difference in staining intensity between IBD patients and control subjects. ROS producing cells were increased in the lamina propria of IBD patients, a fraction of these cells were CD 15 positive. Nitrotyrosine formation was found on ROS positive cells. These results show that iNOS is induced in epithelial cells from patients with active ulcerative colitis or Crohn’s disease. Nitration of proteins was only detected in the ROS producing cells at some distance from the iNOS producing epithelial cells. These findings indicate that tissue damage during active inflammation in IBD patients is probably more related to ROS producing cells than to NO. One may speculate that NO has a protective role when during active inflammation other mucosal defence systems are impaired.

INTRODUCTION

Inflammatory bowel disease (IBD) is characterised by chronic intestinal inflammation. The cellular components of this inflammation are capable to produce reactive oxygen species (ROS), hydrogen peroxide (H$_2$O$_2$), superoxide anions (O$_2^-$), and nitric oxide (NO)\textsuperscript{1}. NO is synthesised from L-arginine by the enzyme nitric oxide synthase (NOS). The constitutive (calcium dependent) isoforms, neuronal NOS (nNOS or bNOS) and endothelial NOS (eNOS), produce small amounts of NO which acts as a neurotransmitter and vasodilator respectively\textsuperscript{2}. The inducible (calcium independent) isoform (iNOS) produces much larger amounts of NO and is only expressed during inflammation. iNOS is induced by cytokines like interferon-gamma (IFN-\(\gamma\)), tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)), interleukin-1 (IL-1) and lipopolysaccharide (LPS). iNOS induction is suppressed by transforming growth factor \(\beta\) (TGF-\(\beta\)), and interleukin -8 and -10 (IL-8,-10)\textsuperscript{3}. These inducer and suppressor cytokines are important in the inflammatory response as present in IBD.
that induce iNOS mediate their effects to a large extent via the transcription factor NF-κB. Steroids inhibit the induction of iNOS and NF-κB.

NO can be directly cytotoxic but can also react with superoxide anions (O$_2^-$) yielding the oxidizing agent peroxynitrite (ONOO$^-$). Peroxynitrite can cause tissue damage by lipid peroxidation, oxidation of sulfhydryl groups of proteins, and nitration of aromatic amino acids like tyrosine, yielding nitrotyrosine. Nitration of tyrosine residues may lead to loss of protein structure and function.

Many manifestations of IBD, including mucosal vasodilatation, enhanced vascular and epithelial permeability and disturbed motility, are consistent with direct or indirect biological effects of NO. There is growing evidence that NO production is enhanced in IBD. End metabolites of the L-arginine-NO pathway like citrulline and nitrite were increased in blood, urine, and rectal dialysates of ulcerative colitis (UC) patients with active inflammation. In UC patients NO could also be detected directly in gas from the colon lumen. Although early studies only found an increased mucosal NOS activity in UC later studies also showed an increased mucosal NO production in Crohn’s disease (CD).

The source and function of the enhanced NO production in IBD is unclear. Considering the beneficial effect of iNOS inhibitors (steroids, anti-TNF-α, IL-10, anti-sense NF-κB) in IBD one could postulate that NO is harmful. However, animal studies using NOS inhibitors and iNOS knock out mice showed an aggravation of inflammation. The aim of the present study was to identify the cellular source and distribution of the iNOS and eNOS isoforms in mucosal biopsies obtained from patients with active IBD. Considering the possible interaction between NO and superoxide anions we also investigated the distribution of superoxide anion producing cells and nitrotyrosine.

**MATERIALS AND METHODS**

**Subjects**

Biopsy specimens were obtained from patients undergoing colonoscopy or sigmoidoscopy. Clinical information about the diagnosis, medication, current clinical status, colonoscopic findings, and results of radiological and laboratory investigations were collected using a standard protocol.

Ulcerative colitis (UC) patients comprised 6 men and 4 women; mean age 37 years (range 21-57). Two patients did not have previously documented UC, one of them used aspirin. The remaining 8 patients were known with UC and were referred because of disease activity. Medication consisted of mesalazine in two, prednisolone in one and mesalazine combined with prednisolone in 5 patients. On colonoscopy there were 7 patients with disease extending above the sigmoid colon (extensive UC) and 3 patients with procto-sigmoiditis only (distal UC).
Crohn’s disease (CD) patients comprised 4 men and 6 women; mean age 30 (range 18-49). Three patients were not previously known with CD, one used inhalation steroids and loratidin for asthma and another patient used vigabatrine for epilepsy. Medication of the remaining 7 patients known with CD consisted of prednisolone in one, mesalazine in one, and the combination of prednisolone and mesalazine in five patients. Of these five patients two also used azathioprine, and one patient also used methotrexate. All patients had colitis and one patient also had a terminal ileitis.

Control subjects comprised 4 men and 3 women; mean age 41 (range 33-61) with an irritable bowel syndrome (4), diverticulosis (2) and a solitary rectal ulcer (1). Biopsies were taken from macroscopically normal mucosa and all had normal levels of C-reactive protein.

**Specimen collection**

Biopsies were taken using a standard biopsy forceps during videoendoscopy and were obtained from the rim of ulceration’s or aphthoid lesions if present. Biopsies were also obtained from macroscopic inflamed mucosa. In 2 patients with UC, and 4 patients with CD we also obtained biopsies from macroscopic non-inflamed mucosa. The biopsies were collected in Hank’s balanced salt solution, frozen in isopentane and stored at -80 °C.

**Nitric Oxide Synthase detection**

Cryosections (4 µm) were fixed in 4% paraformaldehyde for 5 minutes. For iNOS detection, an affinity-purified polyclonal rabbit IgG against the C-terminus amino acids 1135-1153 of human iNOS (cat # SC-649, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a mouse IgG1 monoclonal antibody against amino acids 961-1144 of the C-terminus of mouse iNOS (cat # N39120, Transduction Laboratories, Lexington, KY, USA) were used. For eNOS detection a mouse monoclonal antibody against amino acids 1030-1209 of human eNOS (cat # N30020, Transduction Laboratories, Lexington, KY, USA) was used. Endogenous peroxidase was blocked by treatment with 0.075 % H₂O₂ in phosphate buffered saline (PBS) for 30 minutes. Peroxidase conjugated rabbit antimouse IgG and goat anti rabbit IgG were used as secondary antibodies. The peroxidase activity was developed with 0.2 mg/ml 3-amino-9-ethyl-carbazole (AEC) in sodium acetate buffer containing 0.03 % H₂O₂ for 10 min. Slides were counterstained with haematoxylin and coated with glycerin/gelatin.

**Nitrotyrosine detection**

Cryosections (4 µm) were fixed in acetone for 10 minutes and incubated with an affinity purified rabbit polyclonal antibody (cat # 06-284, Upstate Biotechnology, Lake Placid, NY, USA) against nitrotyrosine. Endogenous peroxidase was blocked by treatment with 0.075 % H₂O₂ in PBS for 30 minutes. Peroxidase conjugated goat anti-rabbit IgG was used as a secondary antibody. The peroxidase activity was
developed with AEC. Slides were counterstained with haematoxylin and coated with glycerin/gelatin.

**Immunohistochemistry controls**

Specificity of staining with antibodies against iNOS, eNOS, and nitrotyrosine was confirmed by 1) performing the immunohistochemical staining with omission of the primary antibodies 2) pre-incubation of the anti-iNOS (Santa Cruz) antibody with the peptide used as immunogen (cat # SC-649 P) and of the nitrotyrosine antibody with 3-nitrotyrosine. The eNOS and iNOS protein fragments used to raise the Transduction Laboratories antibodies against eNOS and iNOS were not available for inhibition studies. Finally, all antibodies were tested on Western-blot using lysates of stimulated mouse macrophages and human endothelial cells as positive controls for iNOS and eNOS respectively.

**Reactive oxygen species detection**

A cytochemical technique specific for superoxide anion production by leucocytes was used. Briefly, cryosections (4 μm) were incubated in a buffer containing 1 mM azide to inhibit endogenous peroxidase activity, diaminobenzidine (DAB) and Mn++. Superoxide (or a derivative) oxidises Mn++ to Mn+++, the latter having the ability to oxidise DAB, resulting in the deposition of an electron dense reaction product. The slides were counterstained with hematoxylin and coated. The number of ROS positive cells was analysed blinded using an Olympus BX50 microscope (Olympus, Japan), a 2 CCD camera (Sony, Japan), and then processed by an image analysis system (Qwin version 2.0, Leica Imaging Systems). The number of ROS positive cells was counted per mm² mucosa. An average of 87, 110, and 117 mm² mucosa per subject was quantitated in the control, UC and CD group respectively. The specificity for superoxide anion detection was checked by performing the staining 1) in absence of manganese 2) in the presence of catalase (500 U/ml) 3) in the presence of superoxide dismutase (300 U/ml). Determination of the identity of ROS positive cells was performed using antibodies against mast cells (AA1, Dako, Glostrup, Denmark), eosinophils (Eq2, Sanbio, Uden, the Netherlands), CD 3, CD 8, CD 10, CD 14, CD 15 (Becton-Dickinson, Bedford MA, USA) and CD 68 (Dako, Glostrup, Denmark).

**Statistics**

The ROS positive cells counts had a normal distribution and accordingly a two tailed Student’s t-test for unpaired data, with Welch’s correction for differences in variance, was applied to determine differences in cell counts between and within subject groups. The cell counts are given as the mean ± the standard error of the mean.
**Figure 1.** Serial sections of a mucosal biopsy from a patient with Crohn’s disease (A-E) and a control subject (F-J). Haematoxylin and eosin staining shows inflamed mucosa (A, arrow at crypt abscesses) and mucosa from the control subject (F). Anti-iNOS staining shows intense epithelial staining in the inflamed mucosa (B) without staining in the mucosa of the control subject (G). Anti-eNOS staining shows endothelial staining in the inflamed (C) and in the mucosa of the control subject (H). Staining of reactive oxygen species producing cells showing abundant positive cells in the lamina propria of the Crohn’s disease patient (D) and sparse positive cells in the control subject (I, arrow head). Staining with anti-nitrotyrosine shows positive cells in the inflamed mucosa (E) and the mucosa of the control subject (J).
RESULTS

Detection of iNOS

Inflamed mucosa of all UC and CD patients showed a strong expression of iNOS in the epithelial cells (Fig.1.B). The distribution was focally with more intense staining at the apical sites of the crypts. iNOS expression was located immediately adjacent to ulcerated areas with intense inflammatory cell infiltration in the lamina propria. In 1 control subject, 2 patients with UC and 5 patients with CD a few inflammatory cells in the lamina propria were weakly iNOS positive. Identical staining patterns were observed with the two iNOS antibodies used. Uninflamed mucosa of 2 UC, and 4 CD patients and the mucosa of the control subjects (Fig.1.G) showed no iNOS expression. Omission of the primary antibody as well as pre-incubation of the iNOS antibody from Santa Cruz with the peptide used as immunogen completely abolished all staining. The antibody from Transduction laboratories recognised a single band of approximately 130 KD in a mouse macrophage lysate on Western blot. The antibody from Santa Cruz did not detect the mouse iNOS in the mouse macrophage lysate. This is probably due to the low homology between human and mouse iNOS at the C-terminal region.

Detection of eNOS

eNOS was only present in the endothelium of blood vessels. Immuno-histochemically there was no difference in staining intensity between UC and CD patients or control subjects (Fig.1.C and H). No staining was detectable when the eNOS primary antibody was omitted. The antibody recognised a single band of approximately 140 KD in a human endothelial cell lysate on Western blot.

Reactive oxygen species and nitrotyrosine detection

There was a significant increase (Fig.2.) of ROS producing cells in the inflamed mucosa of UC and CD patients (Fig.1.D) compared to controls (Fig.1.I). The cell counts for the control, UC and CD group were 2.2±0.7, 7.8±1.9, 14.0±3.0 per mm² mucosa respectively. There was no difference (p= 0.89) in the number of ROS producing cells between IBD patients with (10.7±2.8) or without (11.2±2.4) prednisolone.

ROS-staining was reduced by catalase (90 % reduction) and not by SOD indicating that in human gut mucosa this staining technique is not specific for superoxide anion producing cells. Most of the ROS positive cells in IBD patients and control subjects were also positive for nitrotyrosine (Fig.1.E and J). Nitrotyrosine modified proteins were found on the surface of ROS-positive cells. Double staining demonstrated that the ROS-positive cells were negative for Eg2 (eosinophils), AA1 (mast cells), CD 3, CD 8, CD 10, CD 14 and CD 68. A fraction of the ROS-positive cells were CD 15 positive, suggestive for monocytes/granulocytes. Most of the ROS-positive cells were iNOS negative with both iNOS antibodies. Nitrotyrosine could not be
detected on the epithelium. The nitrotyrosine staining of the ROS-positive cells in the lamina propria could be completely blocked by pre-incubation of the nitrotyrosine antibody with 3-nitrotyrosine. In addition, no staining was detectable when the primary antibody was omitted.

**DISCUSSION**

In this study intense iNOS staining was found in the epithelium of inflamed mucosa of both UC and CD patients. This enzyme was expressed focally and could not be detected in non-inflamed mucosa of IBD patients or control subjects. The expression of iNOS in the inflammatory cells of the lamina propria was weak and present in a minority of patients. The immunohistochemical expression of eNOS does not differ between IBD patients and control subjects. These findings demonstrate that during inflammation iNOS is pre-dominantly induced in colonic epithelium and to a much lesser extent in inflammatory cells of the lamina propria. Epithelial expression of iNOS is not specific for IBD since it was also found in diverticulitis.

Singer et al. observed nitrotyrosine formation in the inflamed colonic epithelium. We could not visualise nitrotyrosine-modified proteins in the epithelial layer of the mucosa. Nitrotyrosine was found at the surface of the ROS-positive inflammatory cells in the lamina propria at some distance from the iNOS positive epithelium. Therefore an interaction between superoxide anions produced in the lamina propria and epithelial derived NO is not likely. In a TNBS induced colitis model in rats distinct and separate sites of NO production and nitrotyrosine formation was also observed. Despite the use of two different antibodies we could not detect iNOS in the majority of the nitrotyrosine/ROS-positive cells. In agreement with the findings of Singer et al.
al these nitrotyrosine-positive/iNOS-negative cells were also present in non-inflamed mucosa of UC patients, CD patients and in control subjects. Since nitrotyrosine is a relatively stable end-product of peroxynitrite action, nitrination could have occurred earlier. Alternatively, the expression levels of iNOS in the nitrotyrosine/ROS-positive inflammatory cells could be below the limit of detection.

We found an increased number of ROS-positive cells in the lamina propria of IBD patients. A small fraction of the ROS-positive cells were CD15 positive suggestive for monocytes/granulocytes. Activation of peripheral and intestinal mononuclear cells plays an important role in the pathogenesis of IBD. These mononuclear cells produce ROS that can activate the transcription factor NF-κB. NF-κB in turn induces the expression of pro-inflammatory cytokines and iNOS. However, observed high levels of iNOS-mRNA in both UC and CD patients but could not correlate this with histological demonstration of polymorphonuclear neutrophil (PMN) infiltration.

The function of iNOS-derived epithelial NO is unclear. It could be protective in forming an oxidative barrier to bacterial invasion at the site of mucosal injury. This theory is supported by the finding of an increased damage in acetic acid treated iNOS knock out mice. Also the NOS inhibitor N⁵-nitro-L-arginine (L-NNA) aggravated the course of colitis in acetic acid induced colitis in rats. However, a beneficial effect of NOS inhibition was seen in trinitrobenzene sulfonic acid (TNBS) and acetic acid induced colitis in rats using N⁵-nitro-L-arginine methyl ester (L-NAME) as NOS inhibitor. The beneficial effect of L-NAME only occurred at a low dose, a higher dose enhanced mucosal damage in TNBS induced colitis. Epithelial derived NO has been shown to cause diarrhoea or tissue damage. However, diarrhoea in a chronic colitis model in rhesus monkeys did not improve with more specific iNOS inhibitors like L-N⁶-(1-iminoethyl) lysine (L-NIL) or aminoguanidine. The different effects of NOS inhibitors could be explained by their specificity for the different NOS isoforms, the timing of administration, the dosage, and the animal model used. Altogether the role of NO and NOS inhibitors in human IBD is currently uncertain and the literature is contradictory.

In our study, we demonstrate that mucosal inflammation is associated with induction of iNOS in epithelial cells and to a much lesser extent in inflammatory cells of the lamina propria. The epithelial localisation of iNOS suggests that colonic epithelium is the major source of the increased NO production observed in IBD. Considering the absence of peroxynitrite modified proteins in the iNOS positive epithelial cells and the presence of ROS-positive cells at some distance in the lamina propria, it seems that peroxynitrite is not involved in NO induced tissue damage. What could be the role of NO produced by the abundantly expressed iNOS in mucosal epithelial cells. Is it harmful or protective? Although the data of this study do not allow solving this intriguing question, one could postulate that NO forms a direct line of defence against the colonic microflora in a situation wherein the normal intestinal defence is impaired.
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References


