THE PHYSIOLOGICAL EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE(iNOS) IN THE HUMAN COLON.

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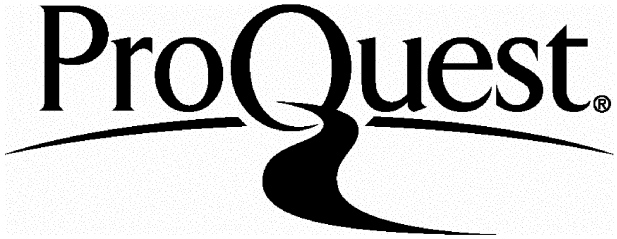

Statement of authenticity

I hereby confirm that all the experiments described in this thesis were designed and performed by myself in the Department of Gastroenterology laboratories, Addenbrookes hospital, Cambridge, UK

Acknowledgments and dedications

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Abstract

Background

Inducible nitric oxide synthase(iNOS) is expressed in the colonic epithelium in both inflammatory bowel disease(IBD) and colorectal cancer.·Nitric oxide(NO·), the product of this enzyme has been implicated in the pathogenesis of both conditions. There is conflicting data, however, on whether iNOS is expressed in the normal, uninflamed human colon.

Hypothesis

In preliminary work using a colonocyte cell line(HT-29), I was able to show expression of iNOS under bacterial lipopolysaccharide(LPS) stimulation. I postulated therefore, that normal human colonic mucosa might express iNOS in the same manner in the light of its close proximity to such stimulants as LPS.

Patients and methods

RT-PCR, immunoblotting and immunohistochemistry were used to investigate iNOS expression in 17 histologically normal specimens obtained at colectomy performed for colorectal neoplasia. In addition, 14 normal mucosal biopsies were obtained endoscopically and evaluated. Twelve surgical specimens and 16 endoscopic biopsies from patients with active ulcerative colitis(UC) were used as inflammatory controls.

Results

All specimens expressed iNOS mRNA, with increased product demonstrated in the inflamed compared to normals using kinetic analysis. Immunoblotting revealed a protein of approximately 130 kDa consistent with iNOS in mucosal extracts of 77% normals and 81% diseased controls. Immunolabelling localised this protein to the surface epithelium

in the majority of the normal specimens and also to the crypt epithelium and inflammatory cells in the colitic specimens.

Conclusions

My data provides evidence that iNOS is commonly expressed in the surface epithelium of the non-inflamed human colon, suggesting that it might be induced by local luminal factors, such as LPS(endotoxin). The resultant $NO^{\cdot}$ produced at this site may act as an oxidative barrier reducing bacterial translocation and providing a means of defence against pathogenic microorganisms.

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Summary

At the time of beginning the work contained in this thesis there were several reports su

ggesting that the inducible isoform of nitric oxide synthase i.e. iNOS is expressed in the colonic epithelium in active IBD(92-95,99-104), and that the resultant nitric oxide(NO·) produced at this site was directly toxic to the colonocyte itself(99), thereby incriminating NO· as a pathogenic factor in this condition. Following on from this, it has been noted that the product of the reaction between NO· and superoxide radical, peroxynitrite (119,120) is also expressed by colonocytes in active IBD and it is postulated that it is this reactive product of NO· oxidation that is cytotoxic in IBD and not NO· itself (99,104). Peroxynitrite will nitrosate tyrosine residues in tissues, forming nitrotyrosine, which can be demonstrated in situ by immunolabelling techniques (126), which have enabled the demonstration of co-expression of iNOS and nitrotyrosine in the epithelium in active IBD(99,104), with the suggestion that tissue damage in this condition is proportional to the amount of nitrotyrosine labeling at this site, an indirect marker of peroxynitrite production. Therefore reports have suggested that increased expression of iNOS and peroxynitrite in IBD is fundamental to the pathogenesis of this condition.

More recent reports have suggested that iNOS could be expressed in a 'constitutive' manner in certain tissues such as the human lung (151), the rat kidney (158), and the mouse

ileum (152). In all these cases there is an absence of tissue inflammation and the authors hypothesize that iNOS is induced by some luminal agent as in each case it is the surface epithelium of these tissues that express iNOS. In the light of these findings I formulated a similar hypothesis that the surface colonocytes in the human colon would express iNOS.

Initial work with the colonocyte cell line 'HT-29' showed that if these cells were grown in a germ free environment then they would not express iNOS mRNA or protein. However when these colonocytes were incubated with bacterial lipopolysaccharide, they were shown to express iNOS mRNA and protein (See Figs1&2a).

In the normal, uninflamed human colon, although there may be an absence of inflammatory cytokines and chemokines, it is plausible that due to continuous contact of the surface epithelium with a variety of luminal agents, then iNOS could be produced in the colonocyte due to these interactions. In the light of this hypothesis I sought to answer the question more thoroughly as to whether iNOS is expressed in the epithelium of the normal human colon.

Using the techniques of RT-PCR, immunohistochemistry and immunoblotting, the expression of iNOS was evaluated in 17 histologically normal surgical specimens obtained at colectomy for colorectal neoplasia, and in 14 endoscopic biopsies taken from patients who were shown either at colonoscopy or barium enema to have normal colons. As it has been shown that carcinogenic factors can induce iNOS(25-28), tissue was obtained from a site distant to the tumour in the colectomy specimens to attempt to avoid such stimulants, and of course the endoscopic specimens were obtained from patients without any adenomas or carcinomas, thus controlling for any such factors.

Another possible stimulus to iNOS expression in the surgical specimens is surgical manipulation itself that undoubtedly occurs during the mobilisation and removal of the

colon. Again the endoscopic biopsies were not subject to such manipulation and 4 full thickness biopsies were taken immediately prior to colectomy, thus avoiding surgical manipulation.

Of the 17 surgical 'normal' specimens, 9 were obtained from patients with left-sided lesions and the remaining 8 from patients with right-sided colonic lesions. The histology of the tumours revealed 14 adenocarcinomas and 3 adenomas with high-grade dysplasia.(see table 1, pg. 43). It is important to note that most of these patients received antibiotics at induction of anaesthesia, but were on no other medications prior to colectomy.

As there is a wealth of data showing iNOS expression in active IBD[92-95,99-104], 12 colectomy specimens taken from patients with refractory UC, and 16 endoscopic biopsies of the same condition were analyzed in a similar manner, acting as inflammatory controls. Most of the patients with UC that went to theatre were on immunosuppressants(table 1.pg 43), and were suffering from pancolitis that had failed to settle on medical therapy. None of the patients were deemed to have toxic dilatation of the colon at the time of surgery.

RT-PCR revealed the presence of iNOS mRNA in all the surgical specimens evaluated (see fig 2a). Kinetic analysis revealed some 6-8 fold increase in iNOS message in the colitic specimens compared to the normal tissues when standardised for G3PDH mRNA levels (see fig 2b). When looking at iNOS protein production, using immunoblotting and a primary anti iNOs antibody monospecific for iNOS, some 13/17(77%) of the normal and 9/11(81%) of the inflamed tissues were shown to express a protein of 130 kDa. This protein band co-migrated with an iNOS protein standard, and was neutralised when the antibody was co-incubated with X2 concentration of the iNOS

peptide, confirming the presence of iNOS protein in the specimens as described (see fig 3).

Further to this, immunohistochemistry localised iNOS protein to the surface epithelium in 14/17(82%) of the histologically normal surgical specimens and in 14/16(88%) of the normal endoscopic biopsies.(see fig 4c). INOS was also expressed in inflammatory cells, as well as the epithelium in 11/12(90%) of the surgical and 15/16(93%) of the endoscopic biopsies of patients with UC(see fig 4g).

Therefore I have shown that iNOS is expressed in a 'constitutive' manner in the epithelium of the normal human colon. This data compliments and extends that of Moochala *et al*(153) who have shown similar data in a smaller number of patients, i.e. 12 endoscopic specimens using the technique of immunohistochemistry alone. A further report suggested iNOS enzyme activity in the mucosa of only a few normal specimens but an absence of iNOS protein expression itself in these specimens (154). This study also suggested that colonic iNOS expression was related to the stage of cancer development with the highest levels in precancerous adenomas. Our surgical specimens were obtained as detailed from patients with colorectal cancer or adenomas although tissue was obtained from a site distant to the lesion. As local carcinogenic factors may play a role in iNOS induction, I also examined colonic tissue taken endoscopically thereby eliminating such effects. All other studies to date, with the exception of the two mentioned above have failed to demonstrate iNOS expression in the normal colon(92-95,99-104). My data has convincingly shown such iNOS production in the colonocytes of the normal human colon in a substantial number of subjects..

Furthermore, it is evident from my experiments that in the normal colon iNOS can be expressed by colonocytes without any evidence of inflammation or cellular necrosis, as judged by standard histological techniques, thereby suggesting fundamental differences

in the factors contributing to iNOS expression in the inflamed, and normal colon. Of course I did not examine the effect of iNOS expression on apoptosis in the colonocyte, another mechanism whereby cellular death can be provoked in the absence of inflammation, with reports of NO· both stimulating and inhibiting apoptosis in a variety of cell systems (183-188). But on the evidence shown in my experiments, iNOS appears to be produced in the surface colonocyte without causing cellular death or necrosis.

The expression of this enzyme in the normal colon is likely to occur in response to some luminal agent such as bacterial LPS or some dietary component, as yet undefined. It can be seen that such expression of iNOS in the surface epithelium of the normal colon is similar to that already described in the respiratory epithelium in man (151). As in the lung, colonocyte iNOS production could be construed as part of the local barrier function of the tissue. The resultant production of NO· from epithelial iNOS activation could provide a means of defence against luminal bacteria, via its ability to provide an oxidative barrier thereby preventing translocation of potentially pathogenic organisms to the deeper layers of the colon (146).

Further effects of local NO· production could be in regulating microvascular patency, permeability, tissue perfusion and mucosal barrier function as discussed above (47,48,64). These effects have principally been shown for the constitutive NOS enzymes but recent work has shown that iNOS induction is critical in maintaining such mucosal homeostasis under conditions of endotoxaemia (145). Currently there is no data describing such effects in the normal colon, but it seems plausible.

Another possible protective effect of colonocyte NO· production could be the effect on cellular apoptosis. In high concentrations NO· will induce 'programmed cell death'-apoptosis in susceptible cells(184), specifically in a colonocyte cell line(185) which can be construed as anti-proliferative. Conversely, low concentrations of NO· seem to protect

certain cells such as endothelial cells (186,187), and human lymphocytes from apoptosis (189). At the cellular level, the chemistry of NO· is ill-defined and complex (189,203), with the potential of NO· to either induce or protect against apoptosis in the normal colonic epithelium, depending upon the luminal and cellular microenvironment.

What about the possible deleterious effects of local NO· production in the normal colon? Apart from the above discussed effects on apoptosis, NO· and its oxides will nitrosate amines (134,135), which in the colonic epithelium would lead to increased levels of luminal nitrosamines with their carcinogenic potential. In addition to this, NO· has been shown to deaminate DNA bases thereby having the ability to alter cellular DNA directly (137,138). Dietary factors are implicated in colorectal carcinoma (200,201), including red meat which increases faecal concentrations of N-nitroso compounds (202). The mechanism by which this increase in nitrosamine concentration occurs is uncertain, but the surmise is that it may be via epithelial iNOS expression, with resultant nitrosation of luminal amines by NO·, thus creating a luminal microenvironment which is more likely to lead to carcinogenesis (202).

I have shown the expression of iNOS in the mucosa of normal human colon. The resultant NO· produced from this enzyme may have beneficial effects by both maintaining epithelial barrier function with prevention of bacterial translocation, and by stimulating apoptosis. Conversely, epithelial NO· if in the right luminal environment may possibly be carcinogenic to the colonic epithelium. Therefore mucosal iNOS expression might be a link between diet or some other luminal component and colorectal carcinogenesis.

Cyclooxygenase is another enzyme that like NOS exhists in both constitutive and inducible isoforms (215-217). It is suggested that the inducible isoform i.e.COX-2 is induced by similar stimulants to iNOS, and that in certain cell systems they are co-inducible(221),

and interact (222,223). COX-2 is upregulated in IBD (218-220), with primarily epithelial expression in man, as opposed to sub-epithelial expression in an animal model of colitis (218). In the light of this I sought to establish whether COX-2 is expressed in a similar physiological manner to iNOS in the human colon. Using the same surgical specimens as already described for iNOS, COX-2 expression was evaluated using RT-PCR, in situ hybridisation and immunohistochemistry. I also looked at 6 specimens from patients with colonic Crohns disease.

All of the inflamed specimens expressed COX-2 mRNA, which was shown by in situ to be localised to neural and smooth muscle cells of the myenteric plexus, inflammatory cells of the lamina propria and the epithelium. Immunohistochemistry confirmed COX-2 protein localisation in myenteric neural cells in all inflamed tissue, with weaker labelling of the colonic epithelium in only half of these specimens. This myenteric neural cell production of COX-2 is an entirely novel finding in IBD and the resultant prostaglandins produced at this site might produce the alterations in colonic motility that are features of exacerbations of this condition. Although COX-2 mRNA could be demonstrated in the normal specimens there was no protein expression seen. Therefore COX-2 is not expressed in the colonic epithelium in the absence of inflammation, demonstrating a fundamental difference as compared to iNOS which I have demonstrated in the normal colon. Another major difference is neural cell expression in the inflamed colon. There are reports of smooth muscle cell iNOS expression in the neuromuscular layers in toxic dilatation in IBD(106), however all the patients in my experiments that were evaluated were deemed not to have toxic dilatation at colectomy and I found no iNOS in the neuromuscular layers in the inflamed samples, again demonstrating a major difference between these two enzyme systems with regards to cellular distribution. Therefore one

might suggest that in the human colon there are infact differing stimulants to iNOS and COX-2 expression which are as yet undetermined.

COX-2 is upregulated in cancer of the colon (227-229), and increased expression in active IBD may explain the increased risk of colorectal cancer in patients with chronic IBD (136,180).

In my thesis I have demonstrated iNOS expression in the epithelium in the non-inflamed colon and have suggested effects of such expression with regards to mucosal barrier function and possible carcinogenesis. I have also shown that in the normal colon iNOS is expressed without resultant tissue damage, although as discussed the effect on colonocyte apoptosis was not examined. Following on from this my data illustrates that although iNOS and COX-2 are said to have similar stimulants for induction, there is a major difference in that COX –2 expression in the human colon is inflammation dependent without any 'constitutive expression of this enzyme system seen. Also it seems evident that neural cells of the myenteric plexus are capable of expressing COX-2 under the appropriate condition of inflammation. This might be the link between mucosal inflammation, prostaglandins and dysmotility. Therefore it appears from my data that there are fundamental differences between iNOS and COX-2 with regards to inducing factors and cellular localisation in the human colon, both in the presence of and absence of inflammation.

Abbreviations.

APS-ammonium persulphate

cDNA-complimentary deoxyribonucleic acid

Ci-Curie(measurement of radioactivity)

DEPC-diethylpyrocarbonate

DTT-dithiotreitol

d-NTPs-deoxynucleotides

α32P-CTP-

DLD-1-human intestinal epithelial cell line

DAB-3'3'diaminobenzidine tetrahydrochloric dihydroate

EDTA-ethylenediaminetetra-acetic acid

ECL-enhanced chemiluminescence

G3PDH-glycerol-3-phosphate dehydrogenase

HT-29-colonic epithelial cell line

IBD-inflammatory bowel disease

IFNα-interferon alpha

IL-1α-interluekin-1 alpha

IEC-6-intestinal epithelial cell-6

KH_2PO-potassium dihydrogen phosphate

L-NAME-N_G-nitro-L-arginine methyl ester

LPS-lipopolysaccharide

$MgCL_2$ –magnesium chloride

NaCL-sodium chloride

Na_2HPO_4-sodium phosphate (disodium phosphate)

NaOH-sodium hydroxide

NOS-nitric oxide synthase: iNOS- inducible NOS

eNOS-endothelial NOS

nNOS-neuronal NOS

NF-κB-transcription factor

NP40(tergitol)-nonylphenoxypolyethoxyethanol

NH_4CL-ammonium chloride

N-methylaminoacetic;N-methyl glycine-sarcosine

PCR(RT)-reverse transcription polymerase chain reaction

PMSF-phenylmethyl sulphonyl fluoride

PBS-phosphate buffered saline

RNA-ribonucleic acid

RIPA buffer(see immunoblotting)

SDS-sodium dodecyl sulphate

TNF-α-tumour necrosis factor alpha

Tris base-(Trizma base)-tris(hydroxymethyl aminomethane)

Tris-HCL-tris-hydrochloric acid

TBS-tris buffered saline

TEMED-N,N,N',N'-tetramethylethylenediamine

Tween-20-polyoxyethylenesorbitan

UC-ulcerative colitis

Chapter 1 Introduction

Historical Background of Nitric Oxide(NO·)

The Discovery of NO·

Nitric Oxide(NO·), an atmospheric gas is one of the smallest known products of mammalian cells and is measured directly as a chemiluminescent product of its reaction with ozone.

The first suggestion that mammals were capable of producing oxides of nitrogen was as long ago as 1916[1] when it was shown using dietary studies of nitrogen balance that more nitrate was excreted than ingested. Further evidence did not follow until it was revealed that rodents produce nitrate in response to endotoxin(bacterial lipopolysaccaharide-LPS) stimulation and that a nine-fold increase in urinary nitrate excretion was shown in humans with infective diarrhoea[2-5].

Further work on rodents showed that murine macrophages could be stimulated to synthesize nitrate under LPS stimulation, conclusive evidence of nitrogen oxide synthesis in mammals[6]. The amino acid L-arginine was shown to be the substrate for this murine nitrite/nitrate biosynthesis and that NG-substituted analogues of L-arginine served as effective competitive inhibitors of this newly documented pathway[7].

Identification of EDRF as NO·

It was shown that vasodilatation of bovine aortic strips induced by acetyl-choline was dependent upon the endothelium, being mediated by a labile, humoral factor termed 'Endothelium Derived Relaxing Factor'(EDRF)(8). The effects of this EDRF were inhibited by haemoglobin and methylene blue, potentiated by superoxide dismutase(SOD)(9), and mediated by the stimulation of cyclic GMP, later shown to be the second messenger of NO·.

Furchgott suggested at the time of this seminal research that EDRF could be NO· and further to this work in separate lines of study firstly Palmer et al(10) and then Ignarro et al 11) were able to show that EDRF was indeed NO·. This led to the identification of L-arginine as the substrate for endothelial NO· production(12) and the confirmation of the role of NO· as the biologically active intermediate of the L-arginine to nitrite/nitrate pathway. This pathway was also shown in activated murine macrophages(13-15).

The Biosynthesis of NO

NO· is the product of 5 electron oxidation of L-arginine catalysed by the enzyme nitric oxide synthase(NOS), a haemoprotein which resembles cytochrome P450 reductase(16) and requires flavine adenine dinucleotide(FAD), flavine mononucleotide(FMN), calmodulin, tetrahydrobiopterin and NADPH as cofactors(17,18).

NOS exhists as three distinct isoforms, representing three distinct gene products(16,19-21) which vary in cellular site, amino acid sequence, regulating mechanisms and hence functional roles. Functionally the NOS isoforms are separated into calcium/calmodulin dependent 'constitutive' isoforms which includes neuronal

NOS(nNOS or NOSI), and endothelial NOS(eNOS or NOSIII), and calcium/calmodulin independent inducible NOS(iNOS or NOSII).

NOSIII is located in the vascular endothelium[22] being mostly membrane bound. NOSI has been localised to the cytosol of both central and peripheral neurons[16], but is also found in extraneuronal sites such as skeletal muscle, the pancreas and the kidney[23,24]. NOSII from now on referred to as iNOS is induced in a variety of mammalian cells by LPS, and an array of cytokines and chemokines such as interleukin-1ß, tumour necrosis factor-α and gamma interferon. Cells capable of expressing iNOS include; neutrophils, macrophages, hepatocytes, endothelial cells, colonocytes and smooth muscle cells[25-28]. As compared to the constitutive isoforms iNOS produces larger and more sustained production of NO· in biological systems[29-31] which explains its role in cell mediated immune responses.

NO·-Physiological effects

It is now known that NO· has pluripotent effects in man, with its three main classical functions being regulation of vascular tone[8], neurotransmission[32] and cell mediated immune response[7,14]. It acts by activating guanylate cyclase with increased cyclic GMP which in turn activates protein kinases.

Effects on cardiovascular system

NO· is well established regulator of vascular tone. Its main effect in the vascular system is that of endothelium dependent relaxation of smooth muscle[8,11,33]. Within the vasculature, NO· is important in the inhibition of both platelet adherence[34] and aggregation[35]. It also inhibits neutrophil chemotaxis[36] and adherence[37]. Therefore, it can be seen that NO· has important homeostatic effects in the cardiovascular system.

Effects on neurological system

NO· is involved in signal transduction in both the central and peripheral nervous systems[16,32], but also in the non-adrenergic, non-cholinergic neuronal system(NANC) of the gastrointestinal and urogenital tracts where such nerves are termed 'nitrergic'[38,39].

NO as an effector of immune function

The first suggestion that the L-arginine/NO· pathway was involved in immune functions in mammalian cells was established in murine macrophages, when it was demonstrated that macrophage cytotoxicity was dependent upon activation of this pathway[7,14].

Macrophage NO· produced via stimulation of iNOS is now recognized as a major effector molecule in host defence in man against a variety of microbes including bacteria, parasites such as malaria and schistosomiasis and a multitude of viruses[40-43]. This system also seems to be important in the tumouricidal activity of macrophages. It is felt that NO· is cytotoxic to tumour cells by a variety of mechanisms. These include its ability to inhibit haem containing enzymes of the mitochondrial respiratory chain such as aconitase of the Krebs cycle, NADPH ubiquinone oxidoreductase and succinate-ubiquinone oxidoreductase of the electron transport chain[15,44], thereby inhibiting cellular respiration. Other enzymes affected are non-haem metalloenzymes, and ribonucleotide reductase with its obvious effects on cellular DNA synthesis[45,46]. Therefore in summary NO· is undoubtedly important as an effector molecule in mammalian cells.

NO· and the gastrointestinal tract(GI tract)

Motility

Neuronal NO·, found within 'nitrergic nerves' of the NANC nervous system of the

Gastro-intestinal(GI) tract[38,39] is of paramount importance in the regulation of motility throughout the GI tract[47,48]. It is involved in the regulation of peristaltic contractions of the distal portion of the oesophagus and relaxation of the lower oesophageal sphincter .In pathological specimens of patients with achalasia and infantile pyloric stenosis, there is an absence of nitrergic nerves and consequent failure of NO· driven relaxation of the lower oesophageal sphincter and the pylorus respectively[49,50].

NO· also stimulates adaptive gastric dilatation, and regulates relaxation of other important sphincter systems in the GI tract such as the ileocaecal valve and the anal sphincter[51]. Lastly, the tonic relaxation of the distal colon is mediated by neuronal NO· synthesis[52]

Maintenance of mucosal defence

Many studies have shown NO· to be a critical mediator of mucosal defence in the GI tract as exhibited by the following:

1.Gastric protection

In rats, NO· donors(chemical substances which release NO·) protect the gastric mucosa from ethanol induced damage. Furthermore, endotoxin treatment affords protection against ingested ethanol in these rats, an effect which is abolished by L-NAME, a non-selective inhibitor of NO· synthesis, demonstrating the protective effects of NO·[53-57].NO· appears to be one of the most important signals for mucus secretion and its obvious physical barrier properties[58,59]. NO· mediates the hyperaemic response to local irritants in the stomach and plays a key role in modulating basal gastric blood flow[60].. NO·also appears to have ulcer healing potential, via effects on epithelial restitution[61-63].

2.Intestinal protection and NO·

Maintenance of intestinal epithelial mucosal barrier function appears to be critically dependent upon NO· [47,48,64], as demonstrated by evidence that NO· maintains epithelial tight junctions and protects against ischaemia-reperfusion damage of the feline intestine[65].

Immunomodulatory effects of NO· in the GI tract.

NO· inhibits mediator release from mast cells in vitro[66,67] and mast cell NO· itself appears to have an immunomodulatory effect via inhibition of other inflammatory mediators such as platelet activating factor[65-68],

NO· also regulates neutrophil recruitment by inhibition of the expression of adhesion molecules, including intercellular adhesion molecule I (ICAM-1) in vascular endothelium[69].

NO· and inflammatory bowel disease(IBD)

There is a growing body of evidence suggesting that overproduction of NO· as a result of iNOS expression could play an important role in the pathogenesis of IBD, a condition characterised by increased local production of many inflammatory mediators[70-75], which are known to induce iNOS [18,25,27,28,76].

Evidence for a pathogenic role for iNOS and NO· in IBD is suggested from cell culture experiments, animal models of colonic inflammation and the use of both selective and non-selective NOS inhibitors in these systems, and human IBD studies.

1.Cell culture systems

Rat colonic epithelial cells express iNOS when challenged with E.coli

LPS and the resultant increased NO· produced was shown to alter colonocyte viability[77]. The same was also shown of a human colonic epithelial cell line HT29, which expresses iNOS mRNA and functional NOS activity as represented by increased nitrite production under cytokine stimulation with TNFα, IFNγ, and IL-1α[74].

Further to this, a small intestinal cell line(IEC-6) exhibits increased NOS activity when stimulated by bacterial LPS and other pro-inflammatory cytokines[78]. More recently, it has been shown that iNOS expression is potentiated by cAMP, an intracellular mediator of several pro-inflammatory agents in a human intestinal cell line, DLD-1[79].

Therefore it is apparent that both colonic and small intestinal epithelial cells are able to express iNOS under appropriate inflammatory conditions.

2.Animal models of IBD

In animal models of colitis there is evidence of increased luminal nitrite and nitrate levels, increased iNOS protein immunoreactivity, and increased calcium independent iNOS activity as shown by increased enzymatic conversion of L-arginine to L-citrulline(the end product of the biosynthetic pathway),[80-85].

3.NOS inhibition in animal models of colitis

Further evidence for a possible pathogenic role for iNOS in colitis is shown by the effects of NOS inhibition in a variety of animal models. Interestingly, the non-specific inhibitor NG-nitro-L-arginine methyl ester(L-NAME) has been effective in reducing intestinal inflammation associated with acetic acid[86], trinitrobenzene sulphonic acid[86,87], and peptidoglycan-polysaccharide[88].

However, it has also been shown that the same inhibitor in similar or different models had some beneficial effects(89), whilst in another set of experiments the inflammation was exacerbated by this inhibitor(90).

The effects of selective iNOS inhibition in similar experiments has also produced discordant results(80,82,88). In summary, these variable effects of NOS inhibitors may be due to the use of differing drug regimes, achieving partial or complete inhibition of constitutive NOS, inducible NOS or both enzymes.

Therefore the benefit of inhibition of iNOS in animal models of colitis has not been categorically demonstrated and of course to date there are no experiments in human IBD patients using selective NOS inhibitors.

4.Human IBD studies

Active IBD is associated with increased levels of various proinflammatory mediators such as IL-1, IL-6, IL-4, platelet activating factor(PAF), leukotienes ,TNFα, IFNγ, prostaglandins and complement(70-75).

Specifically in ulcerative colitis(UC) there is increased mucosal levels of IL-1β, IL-6, TNFα, and IFNγ ,all known 'inducers' of iNOS(18,25-28).

The first evidence of possible increased activity of the L-arginine/NO·/Nitrate biosynthetic pathway in IBD came from the demonstration that rectal dialysates from patients with UC contain raised levels of nitrite, which is a known stable metabolite of NO·, although there was no suggestion of increased production of NO· at this time(91). Middleton *et al*, were the first to demonstrate increased iNOS activity in the colonic mucosa of patients with active UC(92), and following on from this, such increased mucosal activity has now been demonstrated in active IBD in a number of studies(93-95).

In view of these findings there should be increased luminal production of the product of this enzyme activity,i.e. NO·. Indeed this has been shown using a chemiluminescence method(96), infra red diode laser spectroscopy(97) and by using a selective NO· microelectrode(98) in patients with IBD.

Direct evidence for over expression of iNOS protein in active IBD, has been shown repeatedly being localised to the colonic epithelium and inflammatory cells of the mucosa (99-104), and in patients with toxic megacolon(106). It is evident that many of the features of active IBD such as mucosal hyperaemia, vasodilatation, and consequent 'leaky' epithelium are consistent with the biological actions of NO· (65).

NO· and tissue damage in IBD

It is postulated by some workers that over production of iNOS in both Crohns and Ulcerative colitis is critical to the pathogenesis of this disease and tissue injury(99,100,104), although this has by no means been demonstrated conclusively. Mechanisms whereby NO· can cause cellular injury in IBD include:

1.NO· shows great affinity for iron-sulphur clusters which are critical to the function of various enzymes such as glyceraldehyde-3-phosphate dehydrogenase(107), aconitase of the Kreb's cycle(108-110), and a number of electron transport enzymes(108,111-113). The result of NO··binding to these active sites renders the enzyme nonfunctional.

2. NO· induces the release of intracellular iron which inhibits mitochondrial function and DNA synthesis(114-119).

Peroxynitrite and tissue damage in IBD

NO· in the presence of superoxide radical(O_2^-), will produce the highly reactive molecule peroxynitrite, $ONOO^-$(120,121), which indiscriminately attacks biomolecules critical to function and cellular viability(122-124), and has been shown to cause colitis in

rats when administered rectally[125]. Peroxynitrite will nitrosate tyrosine residues to form nitrotyrosine[126] in tissues which can be labeled in situ using specific antibodies. Two groups have exploited this feature by demonstrating a correlation between the degree of tissue injury in IBD and the degree of nitrotyrosine immunolabelling[99,104], suggesting a role for peroxynitrite induced tissue damage in active IBD. There is evidence that NO··and peroxynitrite($ONOO^-$) have different targets in the mitochondrial chain and hence inhibit separate enzymes[127,128].

In summary, although NO· itself is a weak oxidant, peroxynitrite is a highly reactive species and both NO· and $ONOO^-$ themselves or via reactive intermediates may induce cytotoxicity via a variety of mechanism including; tyrosine nitration, lipid peroxidation, DNA strand breaks,and the consequent activation of poly-ADP ribose synthase[122,123,129-132]. Furthermore nitrogen centered oxidants also degrade iron-sulphur clusters of mammalian mitochondrial enzymes, rendering them non-functional.

The carcinogenic potential of NO· in UC

Apart from the potential for cellular toxicity the other possible biological consequence of overproduction of iNOS protein in UC is carcinogenesis. NO·, as discussed, freely reacts with oxygen radicals to yield nitrosating species and the formation of carcinogenic nitrosamines[133-135] which may be important in explaining the increased incidence of colorectal cancer in chronic UC, as high levels of such nitrosamines have been demonstrated in rectal dialysates of patients with active IBD[133]. In addition NO· directly deaminates DNA bases[137,138] thereby creating another possible mutagenic effect in the inflamed colon where the increased levels of NO· might enhance this mutagenic effect in active UC.

The possible anti inflammatory effects of NO in IBD

It can be seen that there is compelling evidence implicating NO· and its reactive products as causing tissue damage in active IBD and furthermore NO· has the potential to produce increased permeability, mucosal dilatation and effect motility, all features of an exacerbation of this condition.

What about possible beneficial effects of NO· in IBD? NO· may diminish epithelial cell damage by scavenging oxygen free radicals and reducing their harmful effects(139). Also due to the ability of NO· to prevent neutrophil adhesion under inflammatory conditions, it will further reduce oxidant stress(37,140,141). This effect on neutrophil adherence is in part mediated by down regulation of ICAM-1 expression on the endothelial surface(70), which may infact be a direct effect of local NO· as supplementation of NO· has been shown to down regulate adhesion molecule expression and thereby leukocyte-endothelial cell interactions (142). Further evidence for an inhibitory role of NO· in inflammation comes from work showing that inhibition of NO· synthesis and function causes an increase in pro-inflammatory cytokines such as IL-6 and TNFα, two such cytokines that up regulate adhesion molecules(143).

Further evidence has shown that NO· can inactivate the transcription factor NF-κB in endothelial cells(144), with resultant down regulation of a whole host of pro-inflammatory mediators. Whether such effects occur in human colonocytes is currently unknown.

NO· is a critical regulator of microvascular patency, permeability, tissue perfusion and mucosal barrier function(47,48,64). Although these functions of NO· are principally mediated by the constitutive NOS enzymes, it has been shown that the maintenance of vascular integrity is iNOS dependent in endotoxaemic intestinal damage in the rat(145). Currently there is no data on the possible effects of iNOS expression and colonic mucosal integrity in humans.

Specific effects of iNOS expression in the colonic mucosa with resultant local i.e. luminal production of NO· might be to provide an oxidative barrier to bacterial invasion(146), thereby preventing translocation of micro-organisms to deeper layers of the colon. Indeed in a recent study it was shown that parental supplementation of L-arginine reduced bacterial translocation through an NO· dependent mechanism(147).

INOS 'Knock-out' murine models of IBD

Advances in recombinant DNA technology have permitted the development of mice that lack the capacity to express the iNOS gene. These animals can produce NO· in a constitutive manner but are unable to 'overproduce' NO· via iNOS induction. In other words, these are iNOS knockout mice(148). These mice would seem ideal for examining the effects of iNOS in experimental colitis but again the data is far from conclusive. On the one hand, a functional iNOS gene seemed to exacerbate the inflammation in TNBS induced colitis in these mice with increased lethality(149). It was shown in these experiments that the absence of a functional iNOS gene conferred a significant resistance to TNBS induced death and colonic damage, and reduced nitrotyrosine formation, suggesting that iNOS is critical to the production of peroxynitrite and colonic inflammation in this model. Also there was an absence of an effect of iNOS on neutrophil endothelial adherence in this model suggesting no role for NO· on this function.

However, in another set of similar experiments it was shown that iNOS does have a protective, anti-inflammatory role in acetic acid induced murine colitis (150). It was suggested from these experiments that the iNOS gene is possibly a normal component of

the inflammatory response and that only under very particular situations does iNOS activity contribute to cellular toxicity. A possible explanation for the differing effects of iNOS gene ablation in these two models of colitis could be the agent used to induce the inflammation. Whereas, TNBS produces transmural changes, acetic acid causes damage confined to the colonic epithelium.

Therefore even with genetic manipulation of iNOS production as seen in these murine models of colitis there is conflicting data as to whether iNOS expression and consequent production of large quantities of $NO^{\cdot}$ is beneficial or in fact deleterious and causing tissue damage.

The constitutive expression of iNOS

There is extensive data suggesting that iNOS requires inflammatory stimuli(cytokine/mitogen, LPS etc.) for its expression , such as exist in IBD. Indeed currently iNOS expression in the human colon is believed to be dependent upon such inflammatory conditions that occur in IBD(92-106). However recent evidence suggests that iNOS can be 'constitutively' expressed in some tissues such as the epithelium of human lung(151), and mouse ileal mucosa(152), with the plausible hypothesis that luminal antigens are the stimulus for iNOS expression at these sites. Whether such 'constitutive' expression occurs in the non-inflamed human colon is uncertain. One previous study demonstrated mucosal iNOS protein expression in normal colon, with an absence of such expression in colorectal cancer(153). Another study showed evidence of calcium independent NOS activity consistent with iNOS in normal colonic mucosa but were unable to demonstrate protein expression when immunohistochemical techniques were

used[154]. All other studies have failed to demonstrate iNOS expression in the non-inflamed colonic mucosa[92-106].

Preliminary colonocyte experiments

In preliminary experiments I found that HT-29 colonocytes would express iNOS mRNA and protein under LPS stimulation. I postulated therefore, that as the colonocyte in vivo is in constant contact with luminal agents such as LPS, it would seem likely then, that the normal human colon would exhibit mucosal iNOS expression.

Aims

To evaluate the hypothesis that iNOS is expressed in the non-inflamed human colonic mucosa, using the techniques of reverse transcription polymerase chain reaction(RT-PCR), immunohistochemistry and immunoblotting in tissue specimens.

CHAPTER 2 Materials and methods.

The following items were purchased from:

Sigma Ltd.

Acetate buffer, acetone, ammonium acetate, ammonium chloride, aprotinin, ß-mercaptoethanol, boric acid, BSA, DEPC, DTT, DAB, EDTA, glycerol, glycine, guanidine thiocyanate, hydrogen peroxide, haematoxylin, HCL, isopropanol, LPS, leupeptin, methanol,n-ethylmaleimide, NP40, potasium dihydrogen phosphate, pepstatin, PMSF, paraformaldehyde, phenol/cholroform5:1, sodium citrate, sarcosine, SDS, sodium chloride,sodium phosphate(disodium phosphate), sodium hydroxide, sodium acetate, sodium azide, sodium deoxycholate, tris base(Trizma), TEMED, tris-HCL, tween-20.

BioRAD Ltd.

APS, Bromophenol blue, TEMED.

Amersham Ltd.

ECL, nitrocellulose membranes, rainbow markers.

Bioline Ltd.

Magnesium chloride, 10X PCR buffer, α32PdCTP, dNTPs.

Gibco UK Ltd.

Molecular weight markers(100bp ladder)

Vecta laborotories Ltd.

AB vectastain.

Scotlab Ltd.

Acrylamide-bis-acrylamide.

Pierce laborotories Ltd.

Immunopure

Antibodies and peptides

Below lists all the antibodies used and their origins.

Santa Cruz Biotechnology, inc.USA.

1.NOS2(N-20): affinity purified rabbit polyclonal antibody raised against human iNOS(see immunohistochemistry text).

2.NOS2 blocking peptide.

3.Anti-rabbit IgG-HRP

DAKO Ltd.

4.Cytokeratin –rabbit polyclonal raised against epithelial cell marker 'cytokeratin'.

5.Biotinylated goat anti-rabbit (2^{nd} layer of ABC peroxidase immunolabelling)

Buffers and stock solutions

PCR homogenisation buffer

DEPC treated sterile water-85mls

Guanidine thiocyanate-64.5g

Sodium citrate-0.97g

ß-Mercaptoethanol-90µls

Sarcosine 0.4mg/ml(added at time of extraction)

PBS(pH 7.4)

Dissolve in 10 Ls distilled water

NaCL-87.9g

KH_2PO_4-2.72g

Na_2HPO_4-11.35g(anhydrous)

Adjust pH with NaOH

TBE(pH 8)

Tris base-55g/L

Boric acid-27.5g/L

EDTA 0.5M-2mls

Adjust to pH with HCL

TBS(pH 8)

Tris-HCL-10mM

NaCL-150mM

Adjust to pH with HCL

Sample (SDS reducing) buffer

Tris-HCL-62.5mM(pH 6.8)

Glycerol-10%(w/v)

SDS-2%(w/v)

Bromophenol blue-0.1%(w/v)

DTT-100mM

EDTA-1mM

Blotting buffer(pH 8.3)

Tris base-5.81g(50mM)

Glycine-2.93g(40mM)

SDS-0.37g(1.3mM)

Methanol-200mls

Made up to 1L with distilled water.

RIPA buffer

Tris-HCL(pH 6.8)-25mM

EDTA-1mM

SDS-0.1%(w/v)

NP40-1%(w/v)SDS-0.1%(w/v)

Sodium deoxycholate-1%(w/v)

NaCL-0.9%(w/v)

5X Running(electrode) buffer

Tris base-15g

Glycine-72g

SDS-5g

Made up to 1L distilled water

diluted X5 (pH8.3)

SDS-PAGE gel

Stacking gel(4% gel, 0.125M Tris-HCL, pH 6.8)

Distilled water-6.1ml

Tris-HCL 0.5M(pH 6.8)-2.5ml

SDS-10%(w/v)-100µls

Acrylamide(30% w/v)/bis-acrylamide(1.034% w/v)-1.3mls

Add just before use:

Ammonium persulphate(10% w/v)-50µls

TEMED-10µls

Total volume of monomer-10mls

Separating gel(7.5% gel, 0.375M Tris-HCL,pH8.8)

Distilled water-4.85mls

Tris-HCL 1.5M, (pH8.8)-2.5mls

SDS-10%(w/v)-100µls

Acrylamide(30% w/v)/bis-acrylamide(1.034% w/v)-2.5mls

Add just before use:

Ammonium persulphate(10% w/v)-50µls

TEMED-5µls

Total volume of monomer-10mls

PAGE GEL-6%

5X TBE-5mls

Distilled water-15mls

Acrylamide(30% w/v)/bis-acrylamide1.034% w/v)-5mls

TEMED-15µls

Ammonium persulphate(10% w/v)-50µls

Cell culture experiments.

HT-29 cells, a human tumour colonocyte cell line was obtained from the European Collection of Animal Cell Cultures. Epithelial cell origin was confirmed by the presence of immunolabelling using a mouse monoclonal anticytokeratin antibody(obtained from Dako Ltd.). HT-29 cells were cultured in McCoy 5(obtained from Sigma Ltd), a specific germ-free media supplemented with 10% fetal calf serum, containing penicillin(10µg/ml and streptomycin(10u/ml). Cells were incubated at 37°C in humidified air containing 5%CO_2. Cells were grown to confluence in 25 ml flasks and passaged up to 5 times with trypsin/ EDTA(0.25%).Viability was assessed using trypan blue exclusion and experiments continued if 80% or more cellular viability was obtained. Stimulation experiments were performed using *E coli.* LPS(20ng/ml, Sigma Ltd) for 6 hours when cells were washed and either spun and frozen at –70C or plated on glass slides and fixed in acetone. Aliquots of frozen colonocytes were evaluated for iNOS mRNA expression using RT-PCR, and the acetone fixed cells were evaluated using immunohistochemistry for iNOS protein expression. Atleast 12 sets of colonocyte stimulation versus unstimulated experiments were performed and analysed as detailed above.

RT-PCR- HT-29 cells.

RNA extraction

Total RNA was purified from frozen HT-29 colonocytes according to the method of Chomczynski and Sacchi(155), involving homogenisation in guanidine thiocyanate solution, acid phenol/chloroform extraction and isopropanol precipitation as described below.Pellets of fresh frozen colonocytes(dissolved in 500µl PBS), were placed on ice in sterile centrifuge tubes and mixed with 5 mls of PCR homogenisation buffer, with 0.4mg/ml sarcosine added to the mixture at this stage. Equal vloumes of acid

phenol/chloroform(pH 4) and 0.5mls of 2M sodium acetate were added, mixed well and left for up to 20 minutes on ice. All centrifugation steps were performed in a bench-top microfuge at 10000 rpm, the initial separation step being performed for 20 minutes. The aqueous supernatant was mixed with an equal volume of acid phenol/chloroform, spun for 20 minutes at 10000 rpm, the aqueous supernatant now being mixed with an equal vloume of isopropanol. This was left mixing at -20°C for 1 hour. The mixture was further spun at 10000rpm for 20 minutes and the supernatant discarded to leave the RNA pellet adherent to the centrifuge tube. This was dissolved in 3 mls of homogenistation buffer, mixed with 3 mls of isopropanol and as before allowed to mix at -20°C for 1 hour. This was then spun at 10000 rpm for 20 minutes and the resultant RNA pellet was washed in 70% ethanol, then dissolved in 300µl of sterile(DEPC) water in a sterile eppendorf tube, and stored at -70°C until analysed.

Reverse transcription

Aliquots of colonocyte RNA(5µg) were reverse transcribed to produce single stranded cDNA with a kit(Invitrogen, USA) using random primers and AMV reverse transcriptase as follows:Frozen RNA samples were thawed on ice,and then spun at 13000 rpm for 10 minutes. The pellet was then washed in 70% ethanol, spun again at the same 13000 rpm for a further 10 minutes and redissolved, after discarding the supernatant in 30µl of streile, DEPC treated water. 5µl aliquots of the RNA solution were taken and to this were added the following(from cDNA cycle kit,Invitrogen, USA):6.5µl of kit water and 1µl of random primer. This was then heated at 65°C for 10 minutes inorder to denature the primer and RNA. After leaving the sample to stand at room temperature, it was spun at 13000 rpm for just 30 seconds. From the cDNA cycle kit as above the following were now added:1µl Rnase, 4µls 5x Reverse Transcription buffer, 1µl 100mM dNTPs, 1µl

80mM sodium pyrophoshate, and lastly 0.5µl AMV reverse transcriptase. The mixture was briefly spun at 13000 rpm and incubated at 42°C for 1 hour. After this period of reverse transcription the samples were incubated at 95°C for 2 minutes to denature RNA-cDNA hybrids. A further period of reverse transcription was performed after adding more AMV reverse transcriptase and incubating at 42°C for 1 hour. After a further 2 minutes incubation at 95°C, 80µls of DEPC water was added to the samples, which were stored at -20°C pending further evaluation. Control experiments were performed as detailed above except with the omission of the reverse transcriptase enzyme.

PCR amplification

Samples of cDNA(50ng) were then analysed by PCR amplification using pairs of oligonucleotide primers designed according to the nucleotide sequences encoding human iNOS(155). They were then synthesized and gel-purified by R&D Systems Europe Ltd.(Abingdon, UK.). Primer sequences were as follows: 5' gAg AAA gCC CCC TgT gCC 3' iNOS sense, 5' TAC CgC TTC CAC CCT ggC 3' iNOS antisense amplifying a 424bp(base pairs) cDNA fragment, spanning exon 2 to exon 6 of human iNOS gene. Synthetic primers specific for human G3PDH were used as an internal standard, having been designed from the reported cDNA sequence(157). These were as follows:5'CAT CAC CAT CTT CCA GGA GC 3' sense, 5'ATG CCA GTG AGC TTC CCG TT 3' antisense, corresponding to codon postions +73 to +231.

Each PCR reaction contained the appropriate volumes of: cDNA sample, 1xNH_4CL PCR buffer(Promega. WI. U.S.A.), 0.8mM $MgCL_2$,O.5µl each primer, O.5mMdNTPs(Ultrapure; Pharmacia. St Albans. U.K.), 1µCi α_{32}P-dCTP(6000Ci/mmol; Amersham Iternational.U.K.), and 2.5 units BioTaq DNA polymerase. Following 5 minutes denaturation at 93°C, hot start reactions were initiated(annealing 60°C 30

seconds, extension 72°C 30 seconds, and denaturation 93°C 30 seconds) and run for up to 45 cycles.

PCR products were analysed by non-denaturing 6% polyacrylamide gel electrophoresis and film autoradiography using radiolabelled DNA size markers(100bp ladder, labelled with α_{32}P-dCTP), the radioactivity being counted using a scintillation counter, enabling verification of densitometric measurements. Products separated in this way were excised and eluted from the gel into O.5M ammonium acetate and ethanol precipitated. The identity of the PCR product was confirmed by direct cycle sequencing using a kit(New England Biolabs sequencing kit, Letchworth, UK), and performed by Dr K. Morgan.

Immunoblotting of iNOS

The monospecificty of the primary iNOS antibody(N20) to be used in immunolocalisation studies was initially assessed by immunoblots. Purified iNOS(NOSII), eNOS(NOSIII) and nNOS(NOSI) obtained from transfected insect cells were used as standards(gift from R. Knowles, Glaxo-Wellcome, Stevenage). No cross reactivity was observed with the antibody and eNOS or nNOS but strong labelling was seen with the iNOS standard. Therefore it was apparent that the primary antibody was monospecific for Inos.

Immunohistochemistry HT-29 colonocytes.

INOS protein expression was evaluated in HT-29 colonocytes using the technique of 3 layer, ABC Vectastain immunoperoxidase labelling, after initially confirming the HT-29 cells as colonic in origin by staining for cytokeratin, a colonic epithelial cell marker. Slides containing HT-29 colonocytes were fixed in acetone and air dried. The slides were

initially washed in buffered PBS and then endogenous peroxidase activity was blocked using an endogenous peroxidase suppressor 'Immunopure'(Pierce Laborotories) applied for 22 minutes. After thorough washing in PBS, 1:20 dilution of normal goat serum was applied to the slides inorder to block non-specific staining. The excess goat serum was blotted from the sections, and not washed with PBS. The primary antibody, a rabbit polyclonal raised against a peptide corresponding to amino acids 3-22 mapping at the amino terminus of human iNOS was applied to the sections at concentrations of 0.5-1μg/ml, dissolved in PBS containing 0.05%BSA and 0.01%sodium azide, and incubated for 1 hour at room temperature or overnight in a 4°C fridge. Following washing in PBS, the secondary antibody, a goat anti-rabbit biotinylated antibody was applied at a concentration of 5μg/ml, dissolved in PBS and 0.05%BSA and incubated for 30 minutes. Another set of washes with PBS was performed followed by the addition of AB vectastain(equal volumes of A, and B Vectastain in PBS with 0.05% BSA ,made up one hour before application) to the sections for 30 minutes. Rinses with PBS were again performed. Sections were then washed in 0.1M acetate buffer for 5 minutes, and incubated in DAB solution made up as follows. DAB(25mg) is first dissolved in 100mls of PBS. To this is added 50-100μls 30% hydrogen peroxide solution which starts the peroxidase reaction and is therefore added immediately to the sections, and incubated for a period of 1-5 minutes, checking for brown staining at regular intervals during the development phase. The sections are then washed in buffer again and counterstained in haematoxylin(1:20), followed by dehydration through graded alcohols and mounting with pertex. Regions of brown stain indicate sites of iNOS immunolabelling. Normal rabbit serum at concentrations up to 2μg/ml was applied on serial sections as a negative control. As an additional control of specificity of labelling, it was ensured that any positive labelling suggesting iNOS protein localisation was neutralised by pre-absorption

of the primary antibody with iNOS peptide, obtained from Santa Cruz, USA(Applied to sections starting at a peptide:antibody concentration of 2:1, dissolved in PBS with 0.05%BSA and 0.01% sodium azide).

Human tissue and iNOS expression

The expression of iNOS mRNA and protein was evaluated in non-inflamed human colon using the techniques of RT-PCR, immunohistochemistry and immunoblotting. Patients with active ulcerative colitis (UC) would serve as inflammatory disease controls. Both endosocpic mucosal biopsy specimens and surgical tissue obtained at colectomy were evaluated for both normals and patients with UC. Ethical approval was obtained and all patients gave consent. Histologically normal tissue was obtained during colectomy from patients with colorectal neoplasia from a site distant to the lesion(n=17). Full thickness biopsies were obtained in 4 of these patients just prior to colectomy, to control for any possible effect of surgical manipulation on iNOS expression. Surgical specimens from patients with refractory UC(n=12) were used as inflammatory controls. All the surgical patients received the same bowel preparation of sodium picosulphate prior to theatre. The characteristics of the surgical patients are listed in table 1. It is not surprising that all the colitic patients were taking heavy immunosuppressive treatment at the time of surgery as these patients were those that had had colitis for variable periods of time(shortest duration of disease 2 months versus longest duration of disease of 18 years), and were deemed to have failed medical treatments. There was however no difference in antibiotic usage in either group immediately prior to their surgery. With regards to the cancer patients left and right sided neoplasms were almost equally represented, as well as varying degrees of tumour activity, which was noted post

colectomy inorder to make any correlation between iNOS expression and tumour type and invasiveness.

Table 1. Characteristics of patient groups Surgical specimens

	Histologically normal tissue	UC specimens
Age(mean years)	55 years(9♂,8♀) (range:40-78 years)	40 years(7♂,5♀) (range:33-60 years)
Medication	Nil	Azathioprine (dose:2.5mg/kg n=7) Methylprednisolone (mean dose:500mg/day n=12) 5-ASA*(dose2.4g/day n=10)
Site of disease	Left sided neoplasm(n=9) Right sided neoplasm(n=8)	Pancolitis (n=12)
Histological diagnosis	Tubulovillus adenoma(n=3) Adenocarcinoma(n=14)	Active (ulcerative colitis)

*5 aminosalicylic acid. ♂-male, ♀-female

Endoscopic biopsies

Histologically normal mucosal biopsies obtained at rigid sigmoidoscopy(n=8) and colonoscopy(n=6) were obtained from patients attending our gastroenterology outpatient department and who were later shown to have normal colons, either on barium enema or full colonoscopy. Obviously those 8 patients attending the clinic for the first time and who had rectal biopsies performed, had unprepared colons, unlike those who went on to

have full colonoscopy, who were given Klean preparation as laxative prior to the procedure. It was felt necessary to analyse both of these types of biopsies as they were not influenced by any local carcinogenic factors or surgical stress, and manipulation, factors known to influence iNOS expression. Inflamed mucosal biopsies from patients with active UC(n=16) served as disease controls. All endoscopic biopsies were frozen in liquid nitrogen and post fixed in fresh 1%(w/v) paraformaldehyde, pending immunohistochemical evaluation. The characteristics of the patients from which the endoscopic biopsies were taken are shown in table2

Characteristics of patient groups-Table 2. Endoscopic specimens

	Normal specimens		UC specimens	
Method of biopsy	Rigid sigmoidoscopy	Colonoscopy	Rigid sigmoidoscopy	Colonoscopy
Age(mean years)	46 years (5♂,3♀) (range 24-73)	46 years (3♂,3♀) (range 24-73)	40 years (3♂,3♀) (range 22-60)	40 years (6♂,4♀) (range 22-60)
Medication	Nil	Nil	Prednisolone (n=4) 5-ASA* (n=6)	Azathioprine (n=3) prednisolone (n=5) 5-ASA* (n=8)
Bowel prep	Nil(n=8)	Klean prep (n=6)	Nil(n=6)	Klean prep (n=10)
Histology	Normal(n=8)	Normal(n=6)	Active colitis No dysplasia	Active colitis No dysplasia

*5 aminosalicylic acid

The patients with UC in the endoscopic group were on more medications than the normal patients, however fewer of the UC patients in this endoscopic biopsy group were taking immunosuppressive therapy unlike their surgical counterparts with refractory disease. More importantly none of the patients who were later shown to have normal colons were on any treatments known to influence iNOS expression.

RT-PCR of colonic tissue

The evaluation of iNOS mRNA in the colonic specimens using RT-PCR was as for the HT-29 colonocytes with some minor changes as described below.

RNA extraction

Total RNA was purified from 200mg of colonic tissue according to the method of Chomczynski and Sacchi[155] described above(page38). Obviously larger volumes of homogenisation buffer(10mls/1g tissue) were required to homogenise the surgical specimens.

Reverse Transcription and PCR amplification

These were as for the colonocytes(see page38-39), but with semi-quantitative PCR analysis being performed on the surgical specimens using kinetic PCR with G3PDH as the 'house-keeper' gene. Kinetic PCR amplification was for up to 45 cycles. Levels of iNOS mRNA were compared to the level of G3PDH mRNA by assessment of transmitted light through the autoradiographic film using a Joyce-Loebl Chromoscan, thereby assessing radiographic band 'densitometry' for each sample.

Extraction and immunoblotting of iNOS in colonic tissue

The monospecificity of the primary iNOS antibody(N20) was shown in initial immunoblots(see pg 40) and the transfected insect recombinant iNOS was used as a positive control for immunoblotting of colonic tissue.

Tissue extraction

Colonic mucosa was homogenised in ice cold RIPA buffer, with the addition of protease inhibitors as follows:PMSF 100μg/ml; aprotinin containing 5-10 trypsin inhibitor units(TIU)/ml diluted to 30μl/ml; pepstatin 1μg/ml; leupeptin 0.5μg/ml and n-ethylmaleimide 625μg/ml. Homogenates(containing equal amounts of protein-20μg) were then solubilised in sample buffer by boiling to 95°C to denature tertiary protein structure, and loaded onto premade 4% stacking SDS PAGE gels and 7.5% separating SDS PAGE gels and electrophoresed in running buffer at100- 200Volts for 45 minutes. An aliquot of the iNOS standard was also loaded in a similar fashion. Rainbow coloured molecular weight markers were also loaded in sample buffer in a similar manner to the samples in order to provide a standard for protein molecular weight, in each gel run. The following incubations were all carried out at room temperature unless stated otherwise.

Separated proteins were transferred to nitrocellulose membrane from the separating gel in blotting buffer at a constant current of 420milliAmps for 1 hour. The membrane was then washed in 0.1% Tween-20/PBS and agitated for 10 minutes. Blocking of possible background, non-specific binding of the primary antibody was achieved by placing the membrane in Tween-20/PBS with 3% BSA for 1 hour (or overnight at 4°C). The membrane was then washed in Tween-20/PBS followed by the application of the primary iNOS antibody at the same concentration as for immunostaining(0.5-2μg/ml) for 1 hour .Further washing in the Tween-20/PBS is followed by probing with the secondary antibody, a goat anti-rabbit linked to horseradish peroxidase, applied at a concentration of 1:5000 for 1 hour. After extensive washing in Tween-20/PBS ensuring that the membrane remained moist at all times, ECL developer solution was applied to the membranes and further analysed using specific chemiluminescence film, applied to the

membranes for a period of 30 seconds to 10 minutes in the dark room. Evident protein bands seen can be compared to the protein standards for molecular weight on the film.

Immunohistochemistry of colonic tissue

The technique of ABC Vectastain immunoperoxidase was used to evaluate iNOS protein expression in colonic tissue and was exactly the same as for HT-29 colonocytes(see page 41). The tissues were however frozen immediately on receipt and then cut into 10μm sections on APES coated slides. These were then fixed in fresh 1%(w/v) paraformaldehyde, washed in PBS and probed for iNOS protein localisation using the method described above.

Statistical analysis

Using a specific programme-StatXact-4(Cytel, Cambridge, Massacheuset, USA), the difference in the proportions of iNOS positive samples in both the normal and diseased groups was analysed with 95% confidence intervals, for both immunohistochemistry and western blotting.Confirmatory analysis was performed using Pearson Chi-square for both groups of samples for these techniques.

Chapter 3. Results

A total of 12 stimulation and 12 unstimulated experiments were performed

On the colonocytes.Decreasing doses of LPS were used, starting at 10 μg/ml with the concentration of 20ng/ml found to be the lowest concentration of stimulant at which iNOS expression was seen in these cells.We were able to demonstrate iNOS expression in all viable HT-29 colonocytes, in all stimulation experiments performed after 6 hours post LPS stimulation both at the level of gene expression(see fig 2a), and protein production(see fig1c).

When analysing the surgical tissues we found iNOS mRNA in all specimens tested and in comparison to the level of G3PDH on kinetic analysis, there was some 6 to 8 fold increase in iNOS mRNA in the colitic as compared to the normal tissue, on densitometry(fig 2b)

Immunoblotting of the surgical specimens revealed that some 76%(13/17) of normal specimens and 81(9/11)% of colitic specimens expressed a 130kDa protein that was seen to co-migrate with the iNOS standard. This protein band was inhibited by the preincubation of the iNOS antibody with X2 concentration of iNOS blocking peptide. Figure 3 is an example of iNOS protein found on immunoblotting in normal tissue with lane 2 being a normal sample showing the presence of a 130kDa protein consistent with iNOS, and lane 1 showing neutralisation of this band on preincubation with the peptide. The percentage of specimens both surgical and endoscopic staining positive for iNOS protein are shown in table 3, page 49, and figure 4c, page 53, demonstrates this iNOS protein reactvity in the normal colon, being localised to the surface epithelium.

Table3. iNOS immunohistochemistry in human colon

	Normal tissue		UC tissue	
	Surgical	Endoscopic	Surgical	Endoscopic
% samples staining positive for iNOS	82(14/17)	88(14/16)	90(10/11)	93(15/16)
Cellular localisation	Surface epithelium	Surface epithelium	Surface and crypt epithelium Inflammatory cells of LP*	Surface and crypt epithelium inflammtory cells of LP*

LP*-lamina propria

Results-Figure1. INOS immunohistochemistry in HT-29 cells

Fig 1a. Unstimulated HT-29-iNOS antibody

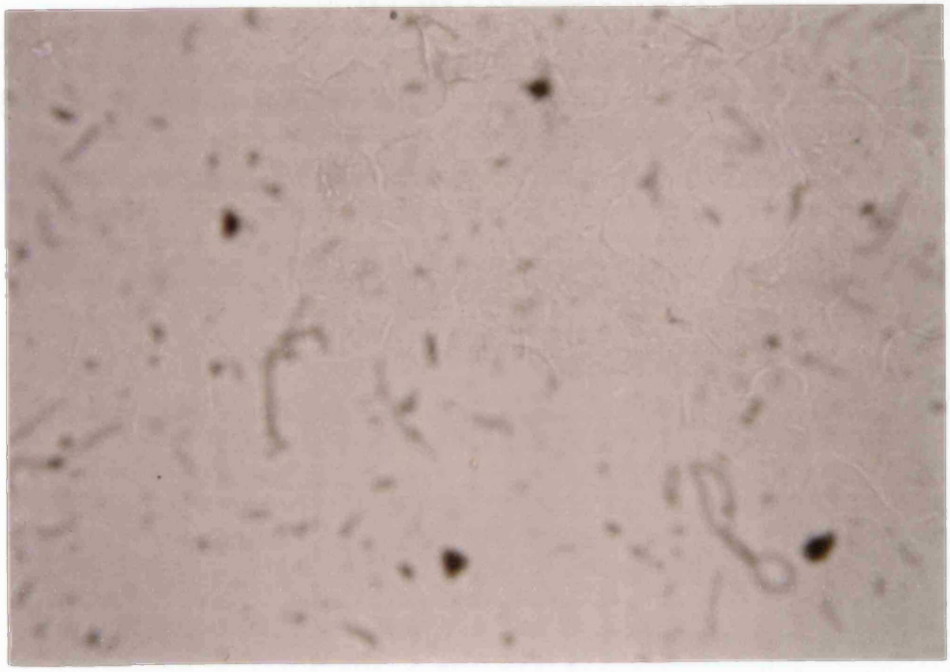


Fig 1b.Stimulated HT-29 –normal rabbit serum

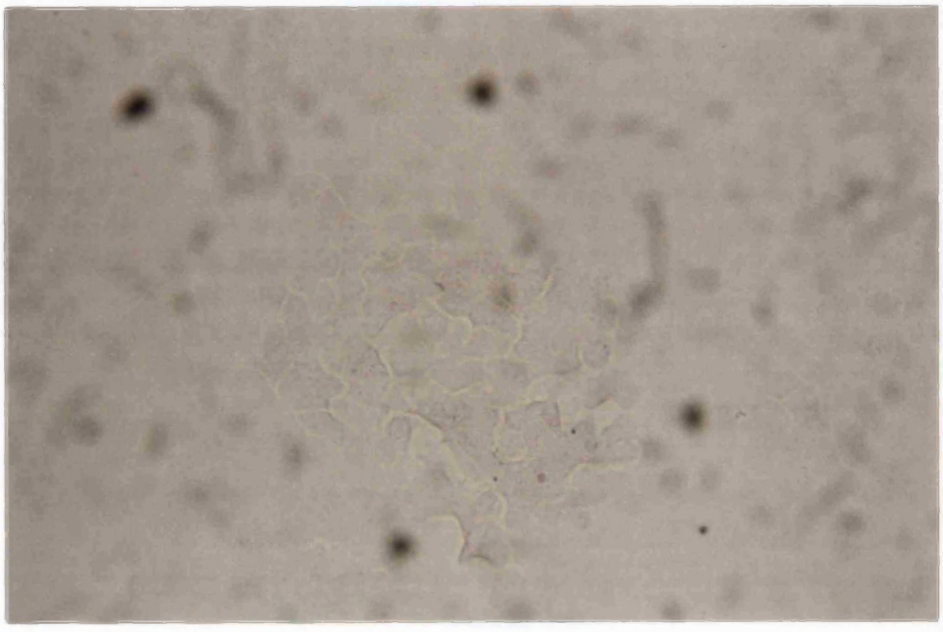


Fig 1c.Stimulated HT-29 cells-iNOS antibody

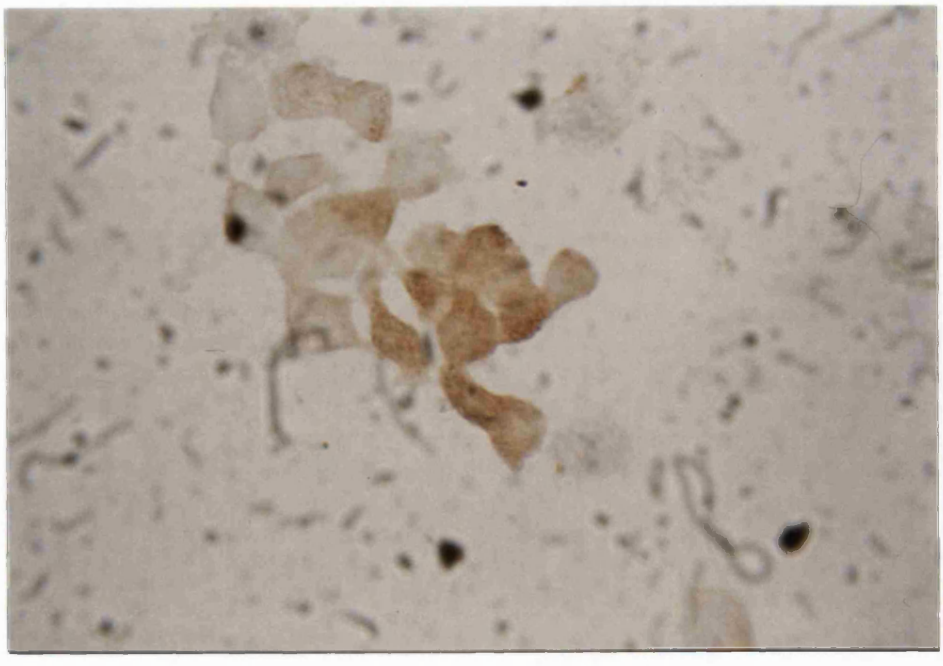


Figure2-iNOS RT-PCR

Fig2a-Qualitative iNOS mRNA expression-surgical tissue/stimulated colonocyte

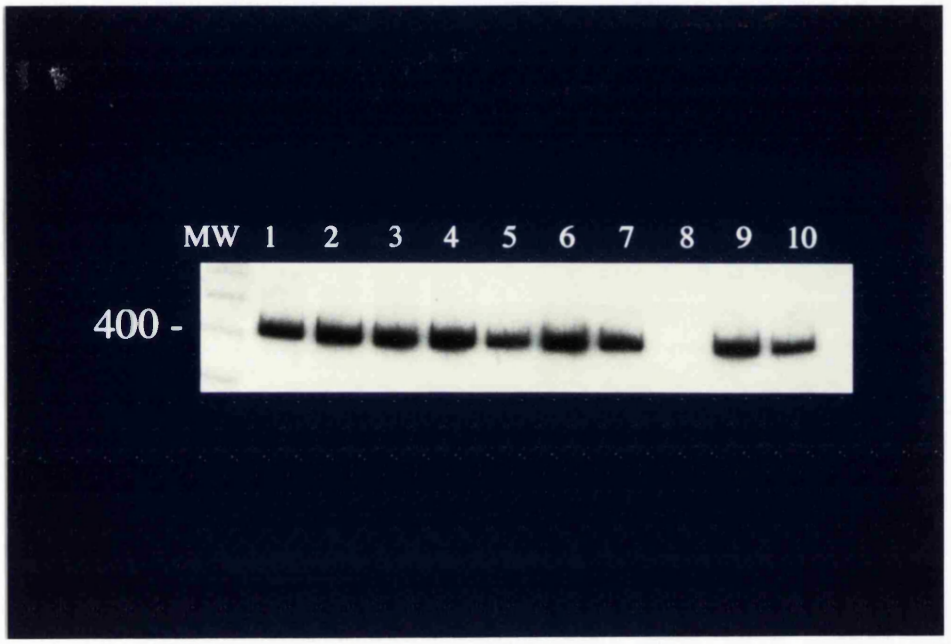


Fig2b-Kinetic iNOS mRNA analysis-surgical tissue

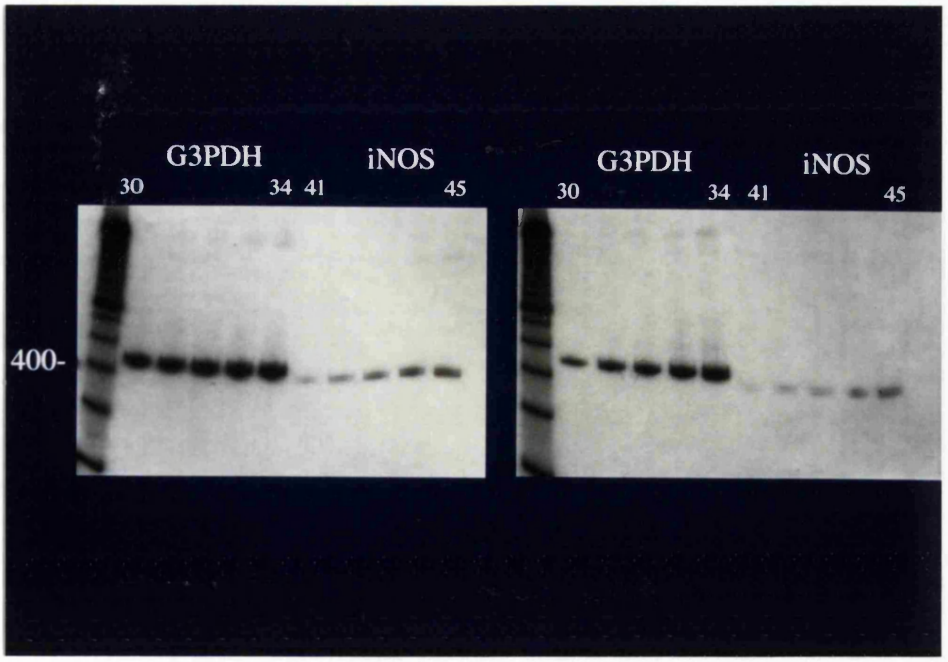


Fig3-iNOS immunoblotting-normal surgical tissue

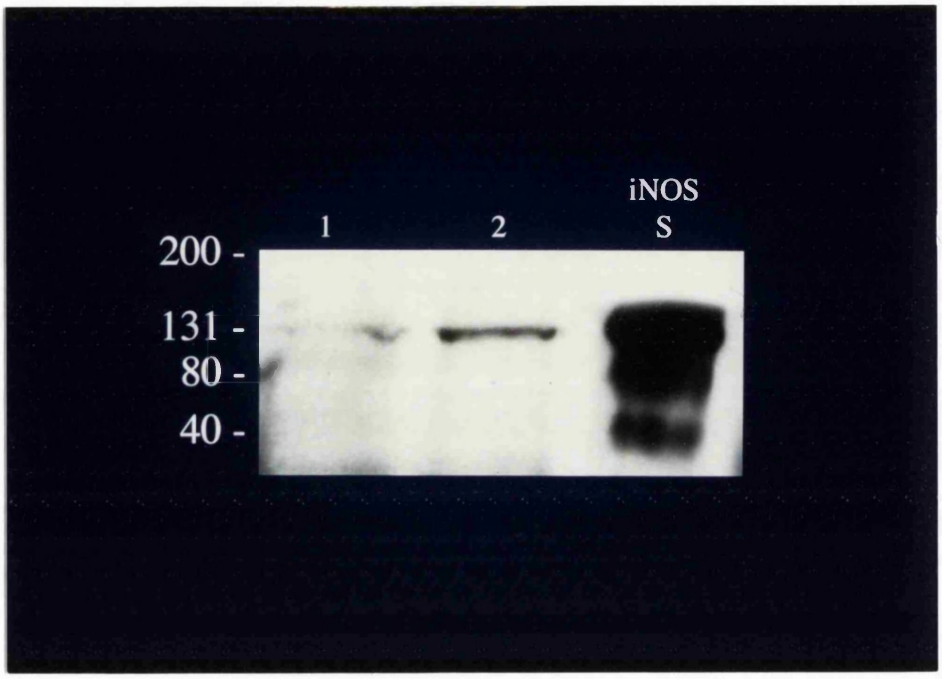


Figure 4-iNOS immunohistochemistry

Figure 4a-H&E-normal tissue

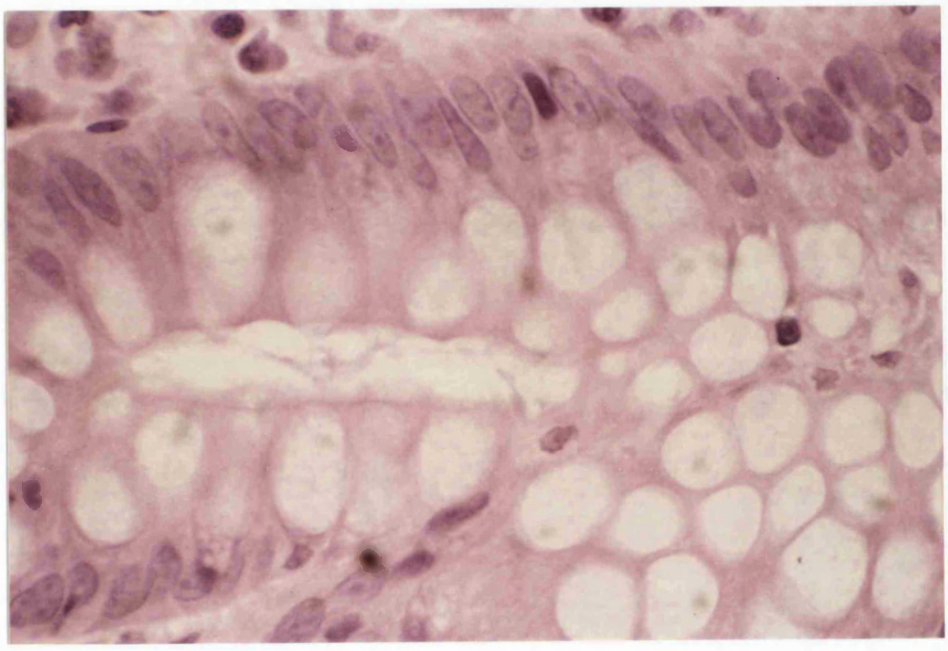


Fig4b- normal rabbit serum- normal tissue

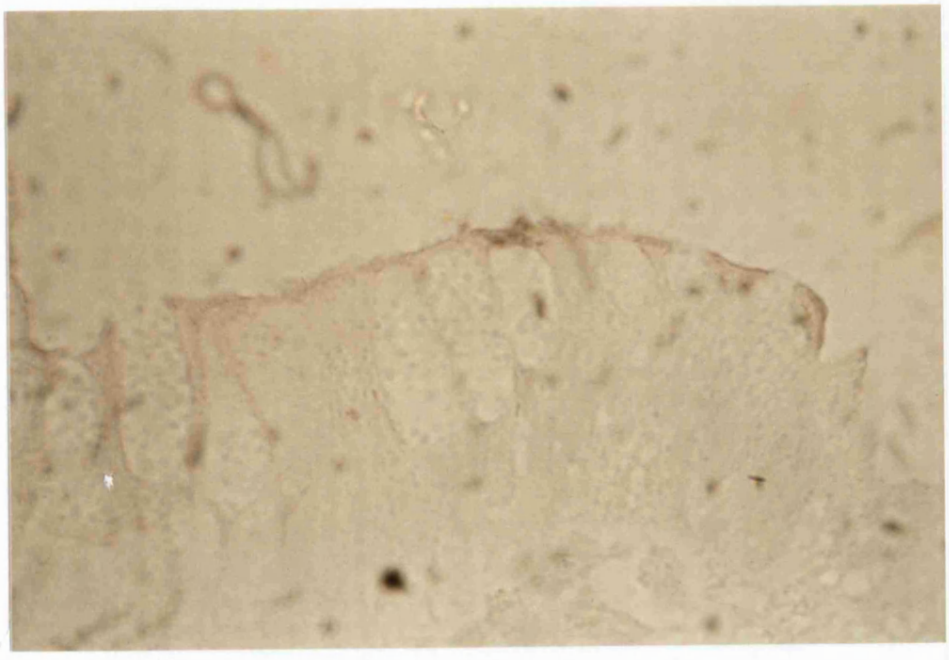


Fig4c-iNOS antibody-normal tissue

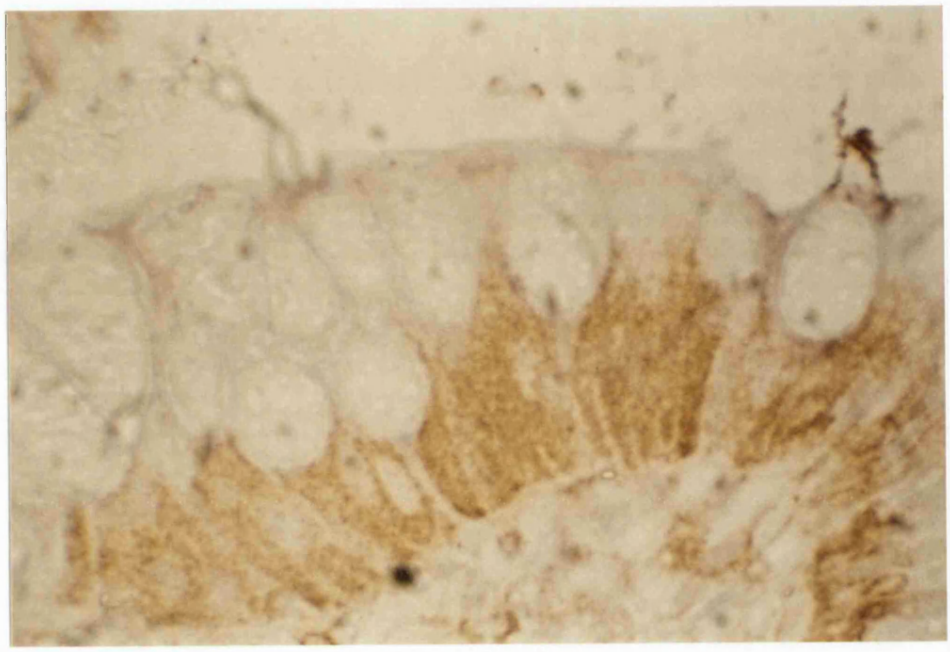


Figure 4d-iNOS antibody/peptide-normal tissue

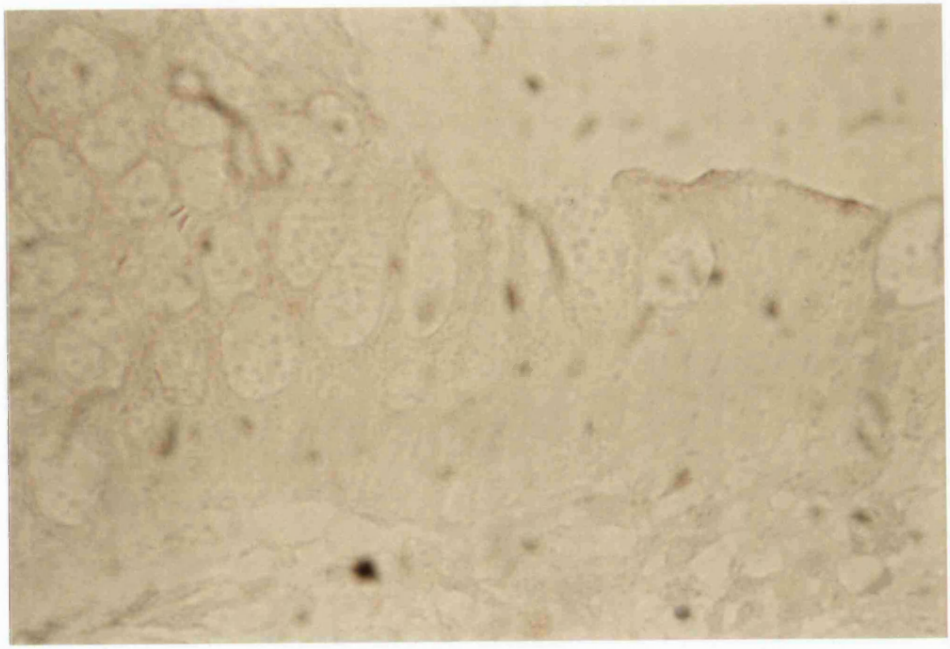


Figure 4e-H&E-colitic tissue

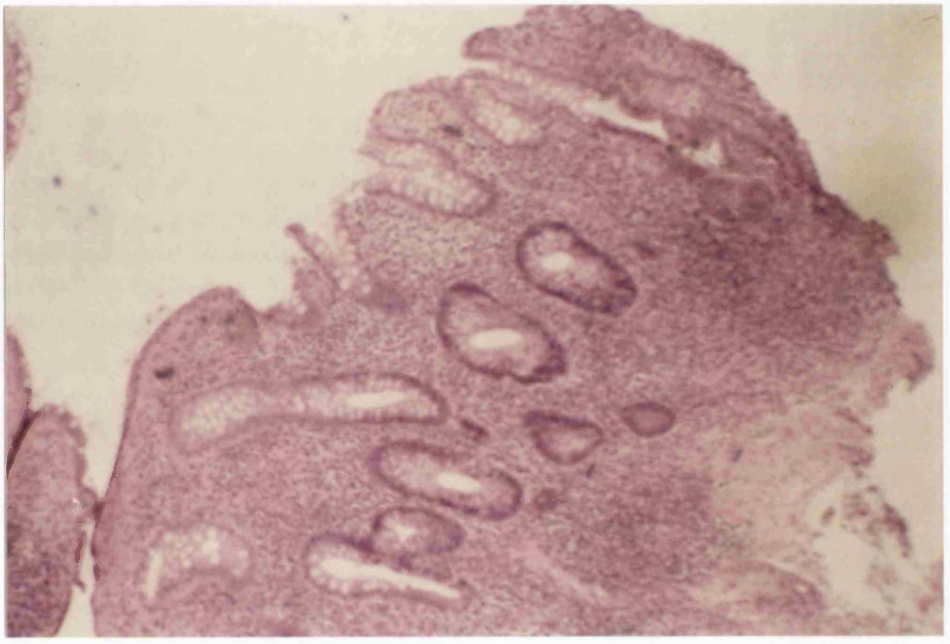


Figure 4f-normal rabbit serum-colitic tissue

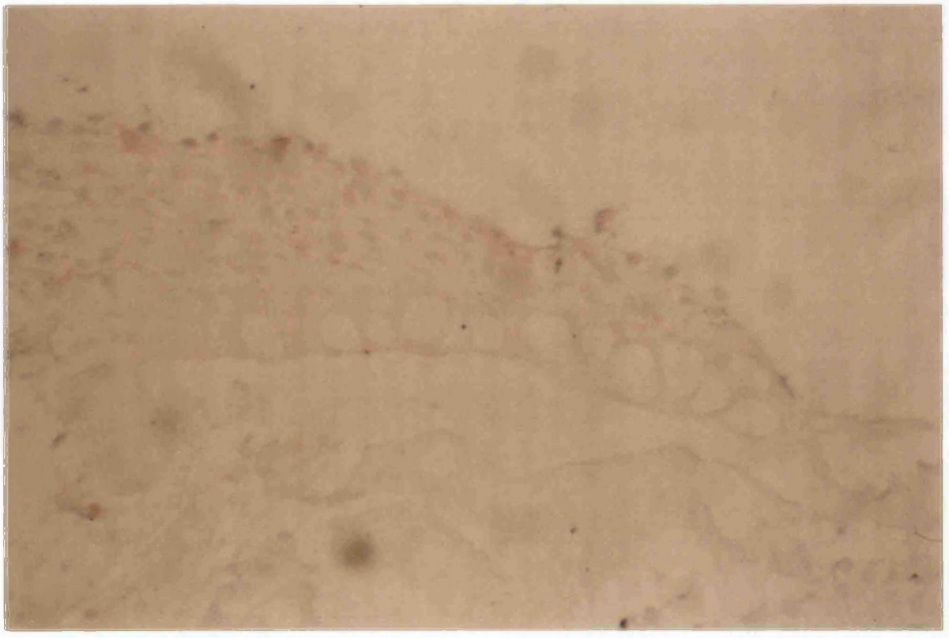
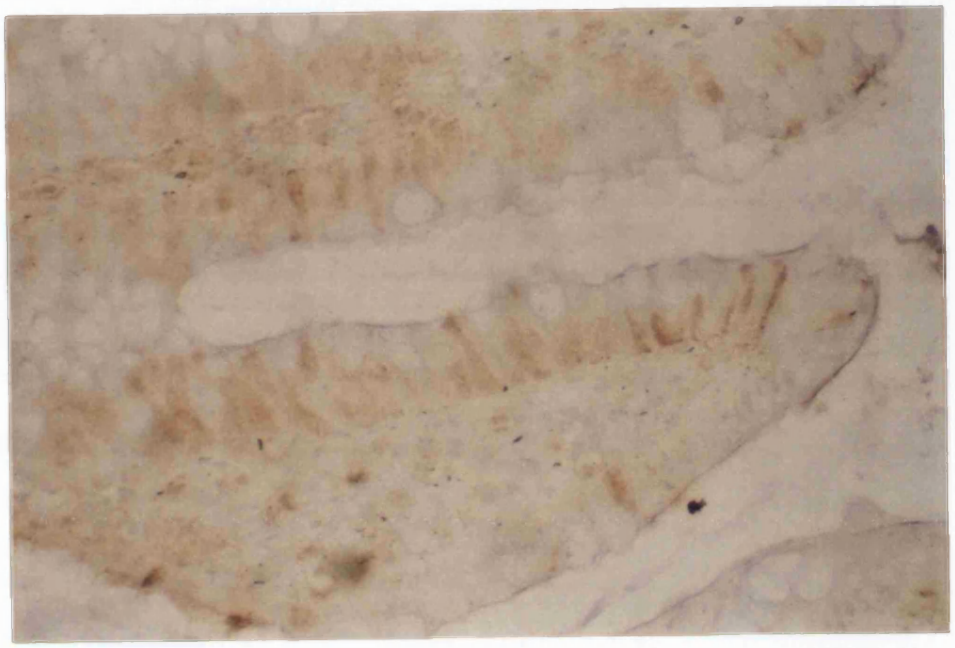


Fig 4g-iNOS antibody-colitic tissue



Statistical analysis

On comparing the proportion of normal and diseased samples that stained positive for iNOS using immunohistochemistry:

95% confidence interval: –0.4138-0.062(p-value-0.14), were found indicating no significant difference in the proprtion of normal and diseased samples staining positive for iNOS protein.

Chi square analysis also revealed non-significant differences in these proportions.

Chi square value-1.127

p-value-0.835

On similar analysis but for western blotting:

955 confidence interval:-0.313-0.48(p-value-0.776).

Chapter 4. Discussion.

My data demonstrates iNOS mRNA and protein expression in HT-29 colonocytes(Figs 1c,&2a) after stimulation with LPS alone, in an otherwise germ free environment. Similar such expression was shown by Kolios *et al*[74] by using a 'cocktail' of proinflammtory cytokines(TNF-α, IFN-γ, and IL-1-α) to induce iNOS expression in HT-29 cells. Further to this Kolios *et al* have now identified those cytokines which have a down-regulatory effect on HT-29 iNOS expression, noteably; IL-13,and IL-4[102].

These prelimenary results and previous reports of colonocyte ability to express iNOS led me to investigate whether intact colonocytes in the normal, non-inflamed colon express iNOS in a similar manner.

My results on human tissue suggest that iNOS is consistently expressed in the normal colon. The surgical specimens were obtained from patients undergoing colectomy for colorectal neoplasia and as a consequence although tissue was taken from a site distant from the lesion, local carcinogenic factors such as cytokines might have played a role in the iNOS expression. Therefore, I also analysed a group of patients who were shown to have completely normal colons, using endoscopically obtained tissue to examine iNOS protein expression and found similar data with the same cellular localisation of iNOS protein. Another problem with the surgical tissue and iNOS expression is that of the effect of surgical manipulation itself. Hence, full thickness biopsies were taken prior to any handling of the colon and analysed using immunohistochemistry. Again similar results were obtained. Therefore it appears that iNOS is expressed, on average in up to 85% of normal colons, with the prime cellular localisation being the surface colonocytes,located in the main to the whole epithelial cell and not just the apical and basolateral borders of the epithelial cell as found in active IBD.These surface epithelial cells are in constant continuum with the luminal flora throughout their viable life. One

could suggest that iNOS is 'constitutively expressed' in the normal colon in the same manner as has been demonstrated in the respiratory epithelium in man(151), the ileal mucosa in the mouse(152),and in the rat kidney(158). My data parallels and extends that of Moochhala *et al*(153) who demonstrated iNOS in the epithelium of normal colon when compared to colonic carcinomas,although the study was limited by small numbers of patients and using immunohistochemistry alone. On the contrary, Ambs *et al*(154) suggested the reverse, i.e. that iNOS expression was most marked in colonic adenomas and carcinomas and concluded that the disparate results obtained were due either to different genetic populations or a lack of specificty of the iNOS antibody used in the initial studies. My immunoblotting data showed that the antibody used in the protein studies was monospecific for iNOS and therefore one would be happy that the staining observed in the normal colon was due to iNOS protein .

My data also confirms many previous reports of increased mucosal iNOS production in active IBD, with similar cellular localisation found in my experiments(92-106), although all these reports have failed to convincingly demonstrate iNOS expression in normal colon, the reasons for which are not apparent and need further analysis. The functional role of increased NO· production in IBD is unknown. There is evidence that NO· is a molecular 'double-edged sword' with both possible protective and damaging roles in this condition.

Firstly, NO· may diminish epithelial damage by scavenging oxygen free radicals that are abundant in IBD(138). Various groups have shown that NO· prevents leucocyte-endothelial cell adhesion in post capillary venules and emigration to the intestinal mucosa, thereby limiting an important source of such oxygen free radicals(37,140,141). This occurs via the down regulation of adhesion molecules such as ICAM-1(70), which has been shown to be a direct effect of NO· on ICAM-1 expression(142).

Tiao[143] showed that inhibition of NO· synthesis and function causes increased production of IL-6 and TNF-α, two cytokines that up regulate adhesion molecules and thereby perpetuate the inflammatory response. Further evidence of the anti-inflammatory effects of NO· is its ability to inactivate NF-κB in endothelial cells[144] via stimulating the production of the inhibitor of this transcription factor. As a consequence of this effect on NF-κβ there follows down regulation of a variety of pro-inflammatory cytokines and consequent salutory effects on the inflammatory cascade. As yet there is no such evidence specifically shown for NO·in human colonocytes but there is obviously the possibility of such an effect in these cells in the presence of inflammation. NO· may also contribute to host defence in conditions such as active IBD by maintaining blood flow to tissues with increased metabolic needs and by modulating epithelial permeability[65]. Horton *et al*[147] demonstrated a benefit of parental supplementation of arginine on bacterial translocation via a NO· dependent mechanism ,all features of NO· that could be construed as beneficial to colonocyte homeostasis under the stress of inflammation.

Data using NOS inhibitors in animal models of colitis in some cases suggest a deleterious effect. For example Pfeiffer *et al* [90] showed that the administration of the non-specific NOS inhibitor L-NAME exacerbated TNBS induced colitis in rats, suggesting an anti-inflammatory role for NO· in this setting. However, other studies using NOS inhibitors have given differing results and are discussed later. The above described effects of NO· relate to inflammatory states such as occurs in IBD. Obviously in the non-inflamed colon such actions of NO· would not be expected to occur. What could be the possible benefit then of epithelial iNOS induction in the normal colon as shown by my studies?

Firstly epithelial iNOS induction and local sustained NO· release could provide an oxidative barrier to bacterial invasion and translocation[146]. Increasing data indicates that

NO· exerts important anti-microbial effects against a wide range of microbes(159) such as *Escherichia coli* and *Salmonella sp.*(160) and iNOS deficient mice are highly susceptible to certain bacterial infections including *Listeria monocytogenes*(161), suggesting the paramount importance of iNOS expression as a means of preventing invasive bacterial infection of the colon. More recently, Forte *et al* (162) have shown a marked increase in endogenous nitrate synthesis in patients with confirmed infective gastroenteritis. Although not definite it seems plausible that this increase nitrate is coming from colonocyte iNOS induction , as previously, Kolios *et al* (103) and Islam *et al* (163) showed such epithelial induction in biopsy specimens of patients with acute *Shigella* and *Salmonella* colitis, with the suggestion that NO· is limiting tissue damage in the presence of infection. Therefore it is plausible that colonocyte NO· production not only helps to kill invasive bacteria but also helps to prevent initial invasion. Furthermore, NO· has been shown to regulate microvascular patency, permeability, tissue perfusion and mucosal barrier function as discussed above(47,48,64). These effects are principally mediated by the constitutive NOS but it has been shown by Hutcheson *et al* (145) that the inducible isoform is critical in maintaining the above effects under conditions of endotoxaemia. Therefore it is possible that epithelial iNOS production in the normal human colon may play a role in mucosal homeostasis although there is currently no data to confirm or refute this hypothesis.

However on the other side of the 'sword' the possible cytotoxic potential of NO· in IBD is well recognised with the suggestion that NO· or a reactive intermediate is involved in the pathogenesis of this condition. Reviewing the animal models of colitis and the use of NOS inhibitors there is evidence that both non-selective and selective iNOS inhibtion is beneficial in some models, limiting tissue damage(86-88). There are to

date no studies using NOS inhibitors in human IBD and so the problem of extrapolating the animal data to the human context still remains.

Further interesting work however, has been in assessing the effects of iNOS gene 'knockout' in animal models of colitis. This also, fails to clarify the role of iNOS in such models as in one experiment using acetic acid induced colitis the absence of the iNOS gene was found to exacerbate the colitis and delay healing(150). This experiment was therefore suggesting an anti-inflammatory role for iNOS in colitis, whereas in another, it was shown that the presence of the iNOS gene in TNBS induced colitis was associated with increased lethality and more severe tissue damage(149). The discrepancies in results could be explained in part by the different stimulus inducing the colitis but it is noteworthy that similar controversial roles have been ascribed to iNOS derived NO· in a variety of pathophysiological conditions. While a number of studies have reported that genetic ablation of iNOS may exert beneficial effects in endotoxaemic shock, infection by *Toxoplasma gondii* and autoimmune vasculitis(148,161,164,165), other studies have reported that genetic ablation of iNOS may exacerbate the inflammatory process in endotoxaemia, encephalomyelitis, and tuberculosis(166-168). Obviously further experiments are needed in order to explain these differences, which have important implications for clinical therapeutic strategies based on NOS enzyme inhibition. It can therefore be seen that the lack of understanding of the role of inducible NOS and resultant increased NO· production is not peculiar to IBD but is apparent for a lot of other conditions in man.

Local NO· release in the inflamed colon may contribute directly to mucosal damage mainly via effects on mitochondrial respiration and include:

1. Mitochondrial enzyme inhibition.(due to high affinity for FeS clusters which are critical to the function of various enzymes involved in formation of high energy phosphates including: G3PDH(107); aconitase and ATPase of the Krebs cycle (108-111); and

NADH-ubiquinone oxidoreductase and succinate-ubiquinone oxidoreductase of the electron transport chain(108,111-113). This would have major effects on the production of cellular ATP, and respiration.

2. NO· induces the release of intracellular iron which inhibits mitochondrial function and DNA synthesis(114-119)

3. NO· in cell culture has been shown to disrupt the zonula occludens that regulates barrier function of the mucosa(169), and in contrast to previously described work, Mishima *et al* (170) showed that iNOS knockout mice were more susceptible to endotoxin induced mucosal injury and bacterial translocation compared to their iNOS wild type counterparts.

A lot of data is accumulating suggesting that peroxynitrite and not NO· itself is the cause of tissue damage in IBD. Peroxynitrite is the product of reaction between NO· and superoxide radical(120,121), two moieties that have been shown to be in abundance in active IBD. Peroxynitrite formation in vivo can be shown by the presence of a stable end product, nitrotyrosine, formed by the addition of a nitro group to the benzene ring of tyrosine by peroxynitrite(126). Although NO· is capable of reaching cells more distant to the NO· producing cell(171), the less stable peroxynitrite, and therefore nitrotyrosine formation is limited to a small radius in the close proximity to its original formation(172). Nitrotyrosine can be localised in tissue using immunohistochemical techniques, which have been utilised by two groups who have shown a correlation between nitrotyrosine staining and the degree of inflammation, suggesting that peroxynitrite is the reactive nitrogen species that is cytotoxic in the setting of human IBD(99,104). Using an iNOS knockout mouse model of TNBS induced colitis, Zingarelli *et al*,(149) have recently shown that the activation of iNOS is required for nitrosative and oxidative damage in experimental colitis.

As mentioned, peroxynitrite is a highly reactive species that attacks biomolecules indiscriminately and may induce cytotoxicity via a variety of mechanisms including:tyrosine nitration, lipid peroxidation, DNA strand breaks with consequent activation of PARS-poly-ADP-ribose synthase(122,123,129-132). Furthermore like NO·, peroxynitrite will degrade FeS clusters of mammalian mitochondrial enzymes thereby rendering cells non-functional.(173,174). Therefore in active IBD it is felt that it may be peroxynitrite and not NO· itself that is harmful to tissues. Indeed evidence is now accumulating suggesting that there are different enzyme targets in the mitochondrial chain for NO· and peroxynitrite(127,128). Also Kennedy *et al*(132) have shown that peroxynitrite and not NO· itself increases trans-epithelial permeability by inducing DNA strand breaks that activate the PARS pathway and cause depletion of intracellular energy stores in Caco-2BBe cells(colonocyte cell line).

To date, no studies have shown the presence of nitrotyrosine residues in the normal colon, which is not surprising considering the relative absence of superoxide radical which is inflammation dependent. My experiments also suggested the same observation. Although there was consistent iNOS expression in the normal colonic mucosa , there was an absence of nitrotyrosine staining(data not shown) consistent with the production of iNOS at this site in the absence of superoxide radical. It may be therefore, that in the context of the normal, non-inflamed mucosa the resultant epithelial NO· in the absence of any other reactive species may have differing effects to when it is produced in the setting of mucosal inflammation. Also when analysing the normal specimens, there is no evidence of mucosal damage or inflammation which would suggest that although there is epithelial iNOS production it is not causing tissue damage in these specimens. One could therefore conclude that in the normal human colon, the constitutive expression of iNOS serves a physiological as opposed to a pathophysiological role. Of course no statement

about what is happening at the microcellular level can be made i.e. if cells are undergoing apoptosis under the influence of iNOS induction(see below). Further work is required to examine the effects of epithelial iNOS production at the cellular level.

The carcinogenic potential of NO· in the colon.

As already discussed increased NO· production may contribute to the pathogenesis of a variety of disorders including IBD but also various cancers[175,176]. There is increased iNOS expression and or activity in human gynaecological[177], breast[178], and central nervous system[179] cancers. Moreover, nitrotyrosine accumulation in the inflamed mucosa of patients with IBD[99,104] as discussed previously suggests that NO· production and the formation of peroxynitrite are involved in the pathogenesis of UC, a condition which predisposes to colorectal carcinoma[180]. These observations suggest that iNOS expression may play a role in tumourogenesis in man. NO· has several properties that might enhance carcinogenesis including:NO· is an endothelial growth factor and specifically mediates tumour vascularisation[181,182], and tumour perfusion[183]. What may be more relevant for the normal colon is the effect of local NO· with regards to apoptosis. Nicotera *et al*[184], showed that high concentrations of NO· induce apoptosis in susceptible cells, which has been shown specifically for a colonocyte cell line[185]. On the other hand, low concentrations of NO· seem to protect certain cells from apoptosis such as endothelial cells[186,187], rat hepatocytes[188], and human lymphocytes[189].

Another carcinogenic effect of NO· is its ability to cause DNA damage by nitrosative deamination[136,137], generating further genotoxic by-products including NO_2(nitrogen dioxide) with resultant DNA strand breakage[190], and peroxynitrite which causes further oxidative damage[191], and DNA modifications such as nitration of purines[192].

NO· may also cause DNA modification by generating carcinogenic N-nitrosamines and DNA reactive lipid peroxidation products via peroxynitrite(193). DNA repair enzymes are also affected by NO·(194,195). The resultant damaged DNA triggers accumulation of p53 protein(196,197) and Forrester *et al* (198) have shown that p53 accumulates in human cells exposed to NO·, either exogenously (using NO· donors) or endogenously generated by over expression of iNOS protein. This work further demonstrated that p53 down-regulates iNOS expression by inhibition of iNOS promoter activity.

It can be seen that deamination of DNA by NO· may represent an important endogenous mechanism of genomic alteration, and it is noteworthy that the point mutations encountered frequently in the p53 suppressor gene in human colon tumours are deamination mutations, which can be caused by NO·. This might therefore reflect, the aetiological contribution of NO· to colorectal carcinogenesis. Indeed Ambs *et al*(154) have shown in some interesting experiments that iNOS is highly expressed in colonic adenomas, with a reduction in carcinomas and suggest that NO· is an endogenous carcinogen that either generates or selects for the high frequency of p53 mutations that arise at the transition from adenoma to carcinoma *in situ* (199).

Dietary factors are implicated in colorectal carcinogenesis(200,201), including red meat which has been shown to increase faecal concentrations of N-nitrosocompounds(202) which are known carcinogens. The mechanism by which this increase in nitrosamine concentration occurs is unknown, but may be via epithelial iNOS expression, with resultant nitrosation of luminal amines by NO·, thus creating a luminal microenvironment which is more conducive to carcinogenesis(202).

NO·, has also been shown in other studies to have the potential to protect against oxygen radical-induced lipid peroxidation and DNA damage by scavenging an oxidising intermediate produced from peroxynitrite(171). Undoubtedly NO· chemistry is extremely

complex[171,203] and incompletely understood at the cellular level. Again it is unclear what role iNOS induction if any may play in colorectal carcinogenesis both in the presence and indeed the absence of chronic inflammation.

Summary of discussion

In summary , I have demonstrated the expression of iNOS in the mucosa of normal human colon, with the absence of tissue damage suggesting that NO· does not cause cellular necrosis in this context. The resultant NO· produced from this enzyme may have beneficial effects by maintaining epithelial barrier function with prevention of bacterial translocation, and also by stimulating apoptosis thereby providing an anti-carcinogenic effect.

Conversely, epithelial NO· if in the right luminal environment may be carcinogenic to susceptible colonocytes. In this context, mucosal iNOS production might be a link between a luminal stimulus, such as LPS or some dietary component and colorectal cancer via its deaminating properties, and ability to increase the luminal content of nitrosamines. Further studies are required to identify the stimulus to iNOS production in the epithelium of the normal colon, and to evaluate the effects of iNOS expression with regards to apoptosis and carcinogenesis.

Cyclooxygenase, like iNOS is an enzyme that exhists in both constitutive and inducible isoforms[215-217], with evidence that it is upregulated in both inflammatory bowel disease[218-220] and colorectal carcinoma[227-229]. There is also evidence that these two inducible enzymes interact[222,223] In the light of such data the possible co-expression of iNOS and COX-2 was examined in the human colon as described in chapter 5.

Chapter 5 COX-2 expression in IBD

Abstract

Increased expression of the inducible isoform of cyclo-oxygenase (COX-2) has been found in active inflammatory bowel disease, although its cellular distribution remains uncertain. Reports suggest possible interactions between the two inducible enzymes of COX and NOS, i.e. COX-2 and iNOS.

Aims

To evaluate COX-2 expression in normal colon and active IBD, and its relationship to iNOS expression.

Patients and methods

Using RT-PCR, in situ-hybridisation and immunohistochemistry COX-2 expression was evaluated in 18 colectomy specimens from patients with active IBD(UC-n=12, crohns colitis-n=6) that had failed medical therapy. Histologically normal colon was obtained from 12 patients having resection for colorectal neoplasia and evaluated as above, acting as non-inflamed controls.

Results

All specimens expressed COX-2 mRNA, with some 6 to 8 fold increase in inflamed tissues compared to controls. In situ hybridisation localised this mRNA to myenteric neural cells, surrounding smooth muscle and inflammatory cells of the lamina propria, with some weaker labelling of the surface epithelium. No COX-2 labelling was seen in control tissue. Immunohistochemistry confirmed these sites of COX-2 expression in all the inflamed specimens, with an absence of immunoreactivity in control tissue.

Conclusions

These findings suggest fundamental differences between COX-2 and iNOS expression, with regards to cellular distribution in both the non-inflamed colon and in the colon of

patients with IBD. No COX-2 protein was found in the normal mucosa, and in the inflamed specimens, although there was weak epithelial labelling in up to half of the tissues, the strongest labelling by far was found in neural cells of the myenteric plexus, an as yet unpublished observation. Resultant prostaglandin production at this site may contribute to the dysmotility seen in exacerbations of IBD.

Introduction

Prostaglandins(PGs), thromboxanes and leukotrienes represent the arachidonic acid metabolites that are collectively referred to as 'eicosanoids'. PGs are produced via the cyclo-oxygenase pathway(204), and like $NO^{\cdot}$ have a variety of effects including both pro and anti-inflammatory effects(205-209). They are also of paramount importance in the gastrointestinal tract in regulating water and electrolyte balance, mucus secretion, blood flow and motility(210).

There is evidence of increased synthesis of PGs, namely PGE_2 in the rectal mucosa of patients with active IBD(211-213),and Lauritsen *et al* (214) have shown a correlation between PGE_2 and disease activity.

Cyclo-oxygenase like nitric oxide synthase exhists in both constitutive(COX-1) and cytokine/LPS inducible isoforms(COX-2), (215-217). COX-1 is believed to produce cytoprotective and anti-inflammatory PGs in a constitutive manner, whereas COX-2 is believed to produce pro-inflammatory PGs and thromboxanes under the appropriate stimulus.

There is increased COX-2 expression in both an animal model of colitis(218) and human IBD(219,220), but with conflicting data on cellular localisation. Reuter *et al*(218) demonstrated in a TNBS induced rat model of colitis that COX-2 was primarily

expressed in the sub-epithelium, whereas Singer *et al* (219) showed epithelial expression in the main in human IBD.

It has been suggested that iNOS and COX-2 might be co-inducible in certain cell systems(221) and indeed may interact with effects on either enzyme system with regards to induction and /or activity. Salvemini *et al*(222) suggested that NO· activates the COX enzyme which may occur due to it's affinity for the haem containing centers of the enzyme. Further sets of experiments demonstrated that NO· modulates PG biosynthesis in vivo in both physiological and pathophysiological conditions and that in situations of co-induction of these two enzymes, some products of COX-2 may down regulate the expression of iNOS(223). Therefore it can be seen that there may be close interaction and regulation of these two enzyme systems by the products of their activation.

In view of the similarities between iNOS and COX-2 with regards to stimuli for induction and their possible close interaction, the expression of COX-2 was examined in the human colon in both IBD and the non-inflamed colon, using the techniques of RT-PCR, in situ hybridisation and immunohistochemistry, with specific attention paid to cellular localisation.

Materials and methods

Patients and specimens

The same surgical specimens were taken and processed as for iNOS evaluation. These include:11 UC , 6 Crohns colitis, and 12 of the normal specimens. The characteristics of the patient groups are described in table 1, page 43.The patients with Crohns colitis all had pancolitis and were on similar immunosuppressant regimes to their counterparts with UC.

RT-PCR

COX-2 mRNA was analysed in the same manner as for iNOS using RT-PCR(see page 45). Specific oligonucleotide primers were designed according to the nucleotide sequences encoding human COX-2(224). The primers were synthesized by R&D Systems Ltd.(Abingdon, UK.), and were as follows: 5'gTT CCA CCC gCA gTA CAg 3' sense, and 5'ggA gCg ggA AgA ACT TgC 3' antisense primer, amplifying a 483 bp cDNA product. During the PCR reaction 1.875mM $MgCl_2$ was used and all else remained the same as described on page 43. PCR cycles were run for up to 35 cycles, and semiquantitative PCR data was generated by kinetic analysis and comparison to levels of G3PDH mRNA.

Again as for iNOS, control data was obtained for COX-2 RT-PCR by omitting the reverse transcriptase enzyme during the reverese transcription

stage.

In situ hybridisation

As preliminary RT-PCR data suggested large quantities of COX-2 mRNA expression in the inflamed tissues we considered the possibilty of localising this product using in situ hybridisation techniques.

Fresh frozen tissue(10μm sections) was cut onto APES coated RNAse free slides and post fixed in fresh 1% paraformaldehyde in PBS. Sense and antisense α32P-labelled single stranded cDNA fragments were generated by PCR. Riboprobes were generated using T7 RNA polymerase after making COX-2 cDNA templates by PCR with an appropriate T7 promoter-tagged primer.Sense and antisense- promoter tagged cDNAs were used as templates in the persence of the T7 RNA polymerase(Promega, WI, USA) and radiolabelled rUTP. In this way it was not necessary to insert the riboprobe template into a pasmid vector. Probes were purified by phenol/chloroform extraction and ethanol precipitated, being washed 6 times with 70% ethanol to remove unincorporated nucleotides. The probes were dissolved in DEPC water, denatured at 90°C for 1 minute, cooled on ice and then serially diluted in hybridisation buffer consisting of:50% formamide, 6X SSC, 5X Denhardts solution(containing BSA, ficoll, polyvinyl pyrrolidine), 100μg/ml, and denatured herring sperm DNA and 100μg total RNA from Baker's yeast. Sections were permeabilised by treatment with 0.2% Triton X-100 for 15 minutes and proteinase K(1μg/ml) for 10 minutes at 37°C. Hybridisation was performed with 30μl of probe for each slide under siliconised coverslips at 42°C overnight in humidified chambers. Coverslips were removed in 5X SSC at room temperature and slides were washed at 42°C in 2X SSC, 1X SSC, and O.5X SSC for 20 minutes in each solution. Washed slides were allowed to dry before performing film autoradiography(with Amersham Hyperfilm β-max, 24-72 hour exposure), followed by emulsion autoradiography (2-5 days with Ilford K5 liquid emulsion). Silver grains were developed using Kodal D-19 developer and then fixed in dilute Amfix(Champion, UK). Sections were counterstained, coverslips placed on, viewed and photographed where appropriate.

Immunohistochemistry

COX-2 protein expression was examined in tissue using 3-layer standard ABC vectastain technique as desribed for iNOS (see page 47). The primary antibody, a rabbit polyclonal raised against COX-2 protein(obtained from Cayman Chemical), was applied to sections at a concentration 1:1000 and incubated for 1 hour at room temperature. All other steps as for iNOS immunohistochemistry. Normal rabbit serum was applied as a negative control(concentration up to 2μg/ml). Localisation of COX-2 was compared to that of the neural cell marker, protein G peptide(PGP9.5) and a macrophage marker-CD68, using appropriate antibodies(obtained from DAKO ltd).

Results

Using RT-PCR all specimens were shown to express COX-2 mRNA as illustrated in figure 5a.In figure 5b it can be seen that kinetic analysis revealed increased COX-2 mRNA product in the diseased specimen(lane2) compared to the normal (lane 1).Indeed there was up to 8X COX-2 mRNA in the inflamed as compared to normals on densitometry, when standardised to G3PDH levels. In situ hybridisation localised COX-2 mRNA to the myenteric plexus(neural cells and smooth muscle) of the inflamed tissue, with labelling also seen in inflammatory cells of the lamina propria. No COX-2 mRNAwas found in the normal specimens using this technique.(see fig 6c).

Immunohistochemistry localised COX-2 protein to the myenteric neural and smooth muscle cells of the inflamed samples, along with inflammatory cells in a similar manner to that observed using in situ. There was some weak labelling of the surface epithelium in 8/12 of the inflamed tissues which was by no means as intense as that of the myenteric plexus and inflammtory cells.(see fig 7). PGP9.5 was co-expressed at the same sites of COX-2 immunolabelling in the diseased tissue confirming its neural cell expression.

Results

Figure 5-COX-2 RT-PCR

Fig 5a-Qualitative RT-PCR

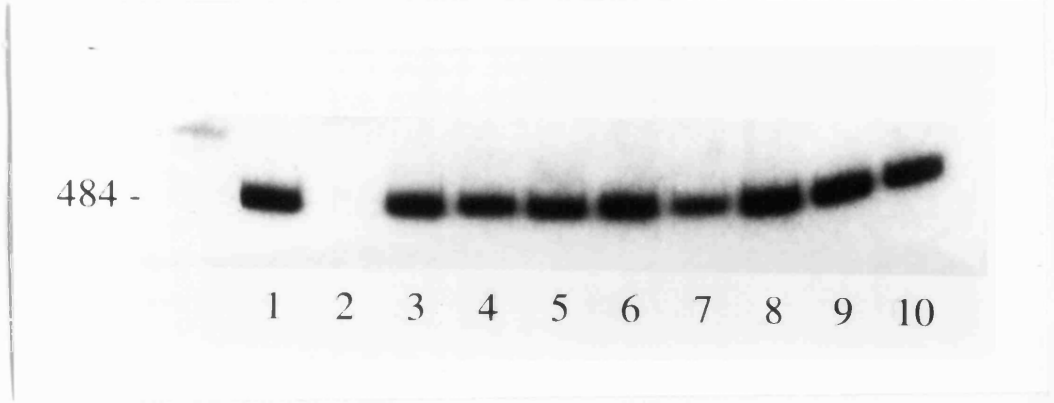


Fig 5b- Kinetic PCR

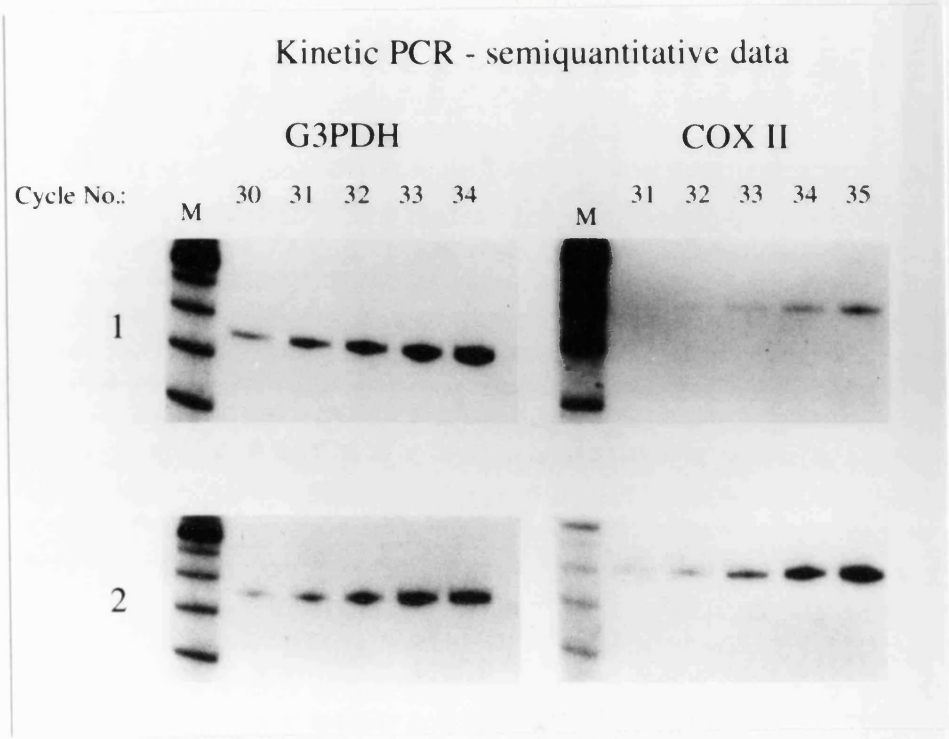


Figure 6-COX-2 in situ hybridisation

Fig 6a-Sense COX-2 probe -inflamed tissue

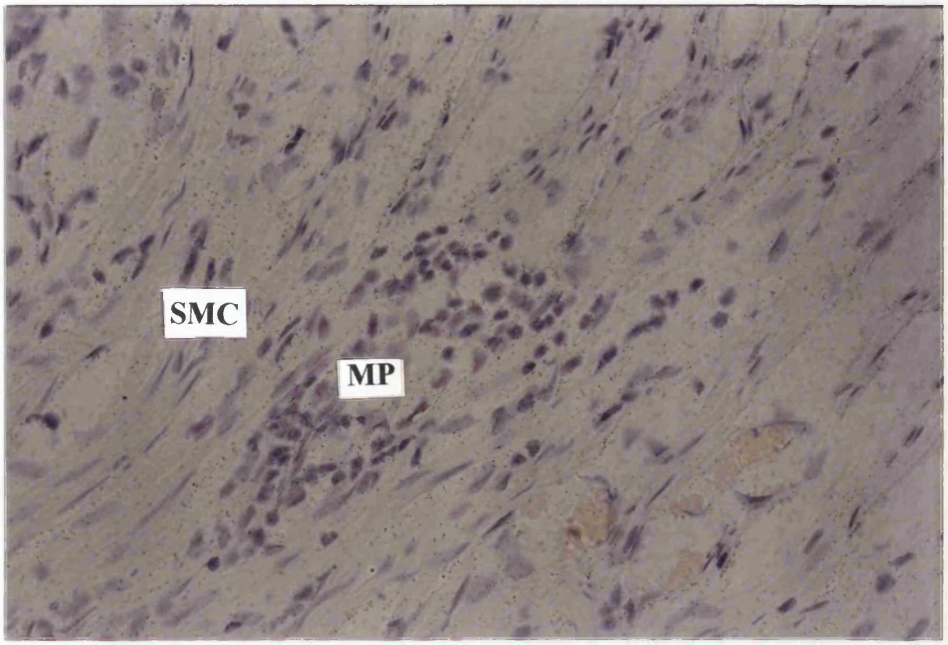


Fig 6b-Antisense COX-2 probe-inflamed tissue

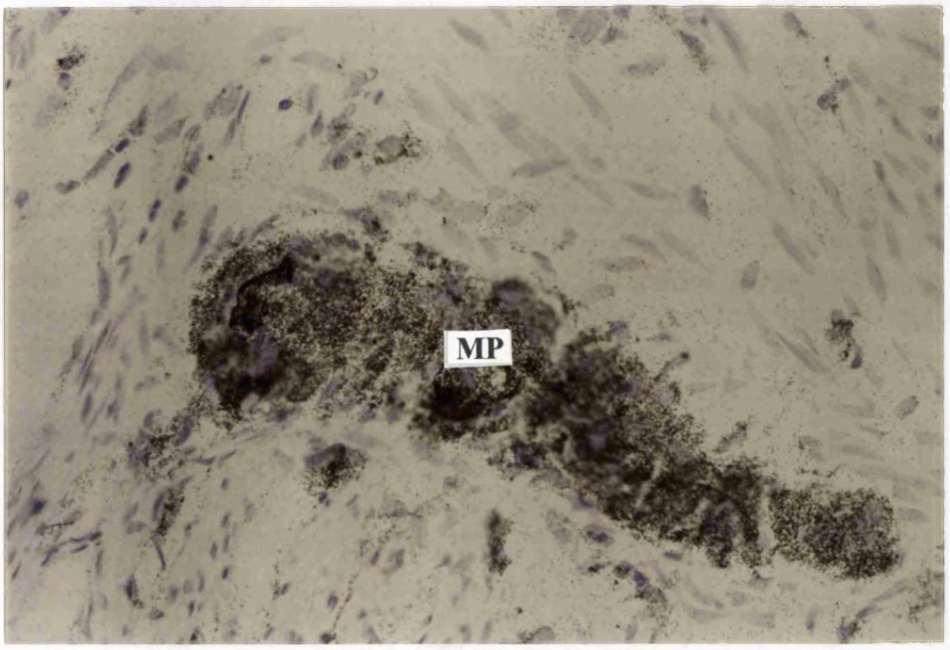


Fig 6c- Antisense COX-2 probe-normal tissue

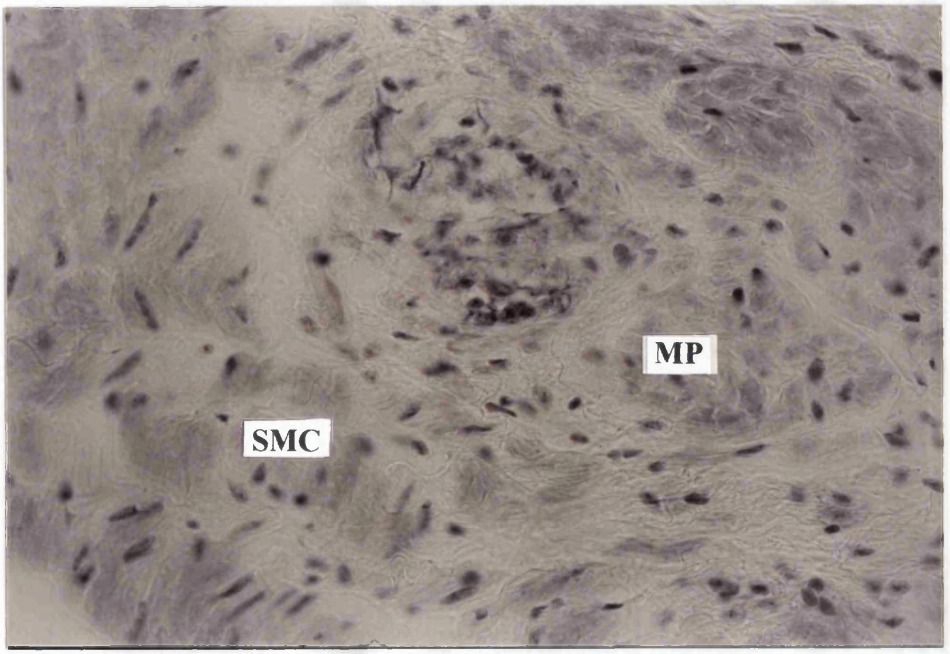


Figure 7-COX-2 Immunohistochemistry

Fig 7a- haematoxylin stain-myenteric plexus-inflamed tissue

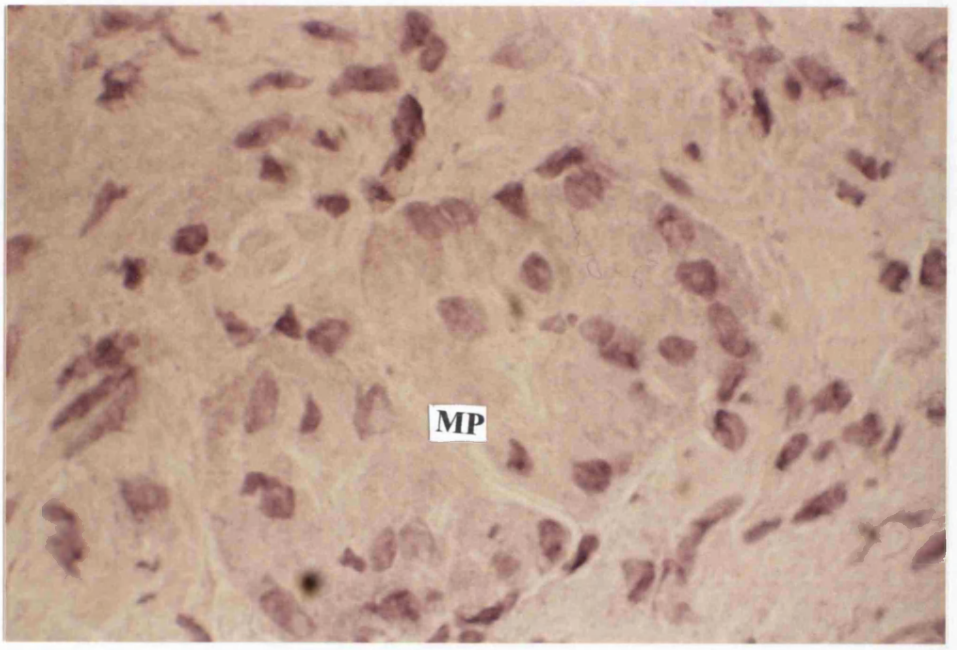


Fig 7b-normal rabbit serum-inflamed tissue

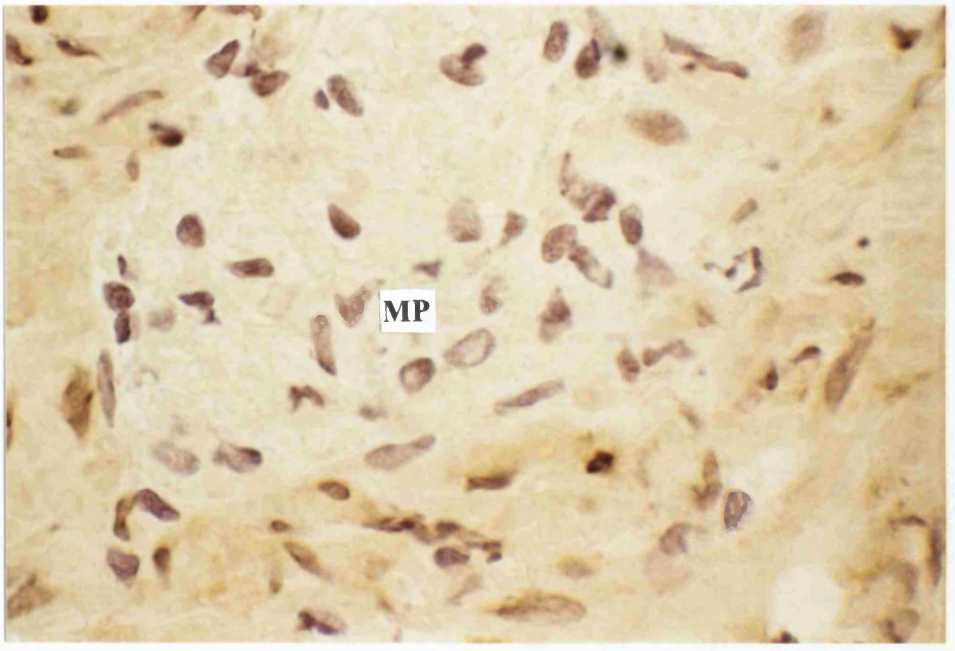
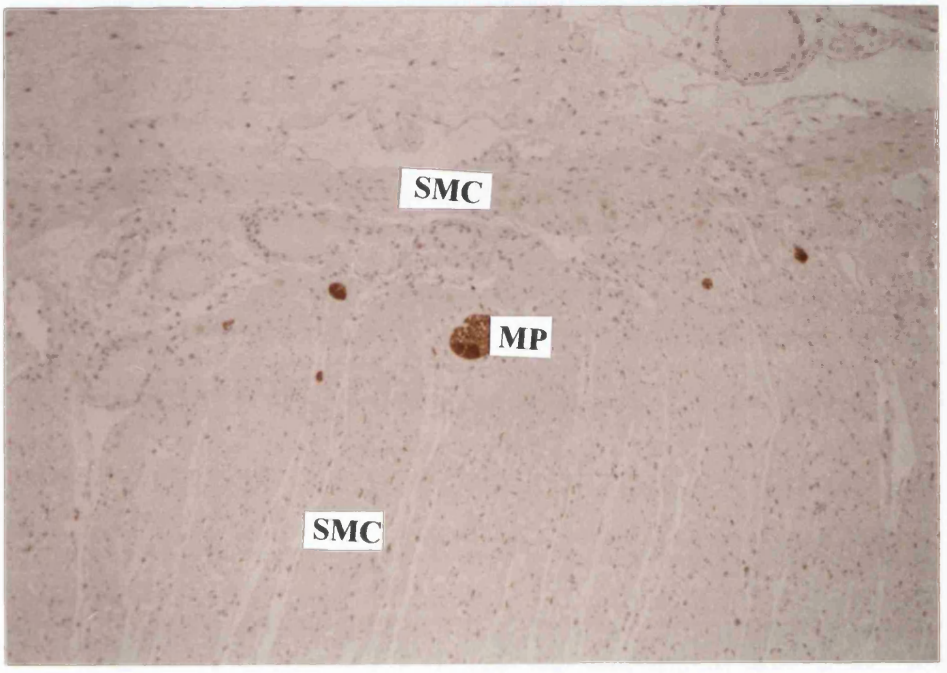


Fig 7c-COX-2 antibody-inflamed tissue

(i)-low power



(ii)-high power view

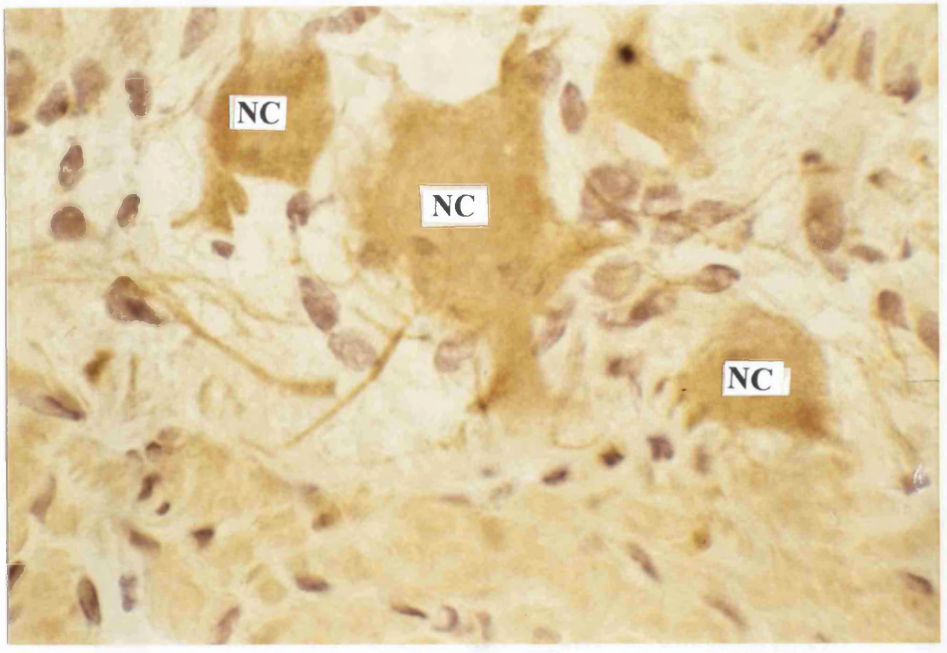


Fig 7d-COX-2 antibody-normal tissue

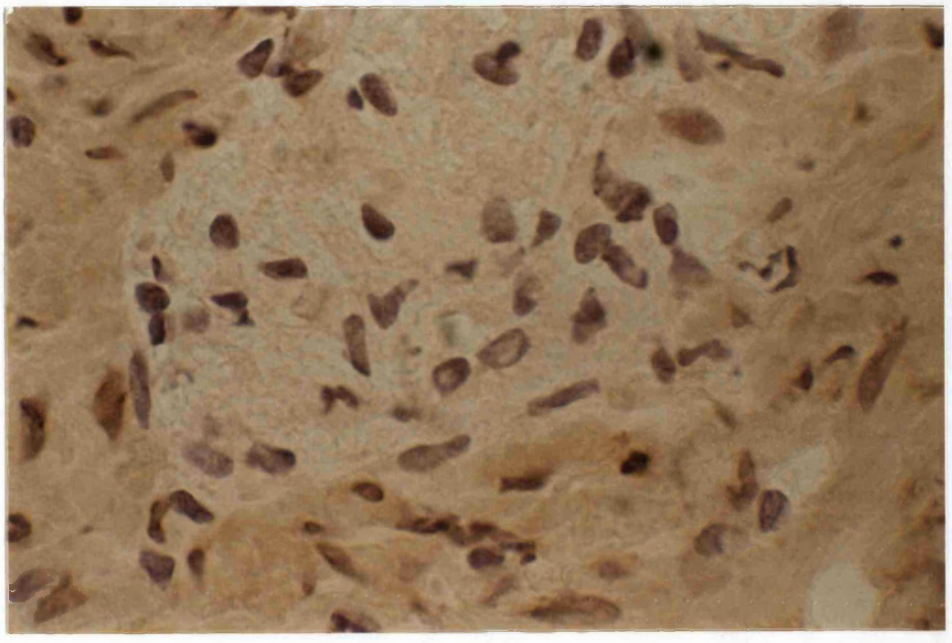


Figure 7e-COX-2 antibody-inflamed mucosa

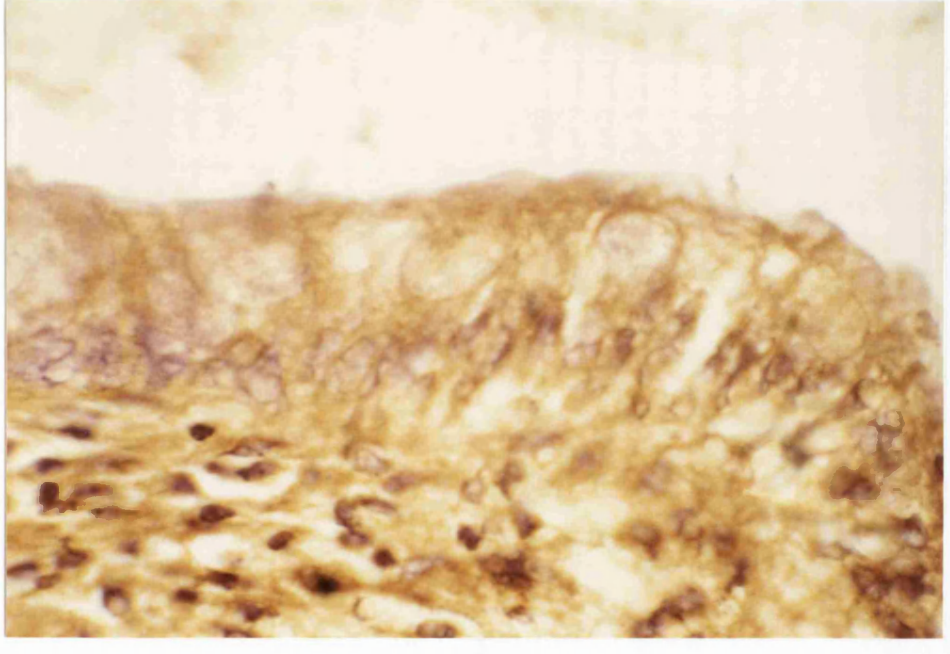
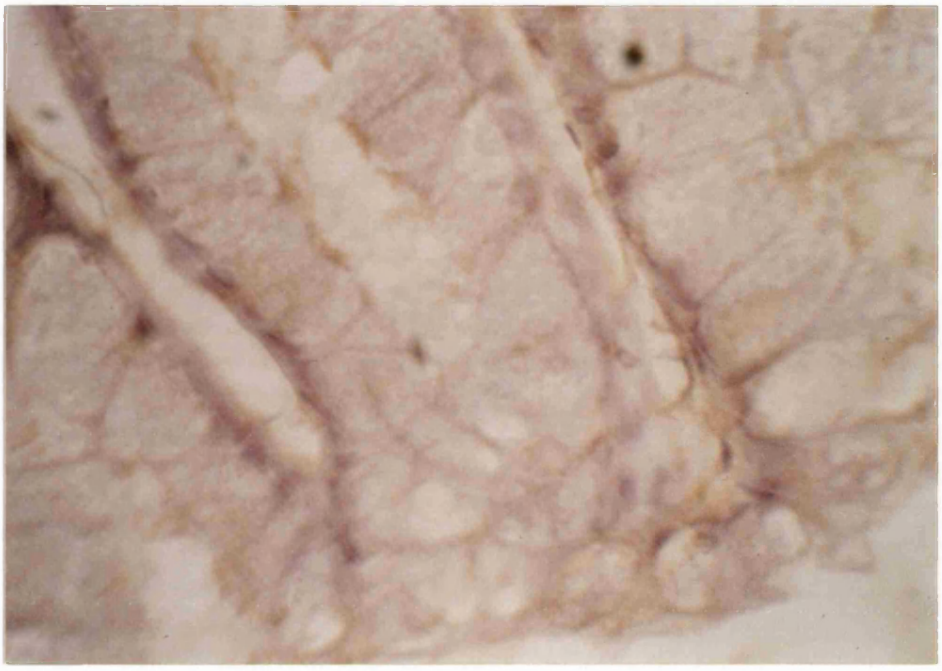


Figure 7f-COX-2 antibody-normal mucosa



Discussion

My data concurs with previous studies showing increased COX-2 expression in active IBD[218-220]. I found, as in one previous report, COX-2 localisation to both the surface epithelium and inflammatory cells[219], although epithelial labelling was by no means consistent or strong. However, I have shown for the first time, intense COX-2 gene and protein expression in both smooth muscle and neural cells of the myenteric plexus of the inflamed colon, with the most striking feature being consistent neural cell labelling. This was found in every inflamed specimen examined, (including both the Crohn's and UC specimens), and using antibodies to both neural cells(PGP9.5) and macrophages(anti-CD-68,) it appears that there is specific neural cell expression of COX-2 in the human myenteric plexus and not activated macrophage expression as suggested in one previous animal study[225].

In certain cell systems, a recognised stimulus for COX-2 expression is mechanical stress which would have occurred during the manipulation and resection of the colonic specimens[225]. However the absence of any COX-2 expression in the normal tissues(using localisation techniques) would suggest that such physical effects have no role in the expression of COX-2 in our specimens, as all tissue examined was obtained and dealt with in a similar manner(i.e.resection at laporotomy).

Another possible bias is that the normal tissues, although taken from a site distant from the neoplasm are nevertheless, from patients with colorectal neoplasia, a known stimulus to COX-2 induction[227-229]. We found some COX-2 mRNA in the normal specimens, consistent with previous reports. However, we found no COX-2 protein in normal specimens which is in contrast to colorectal tumours which express high levels of protein[227-229]. Therefore the absence of COX-2 protein expression in the normal tissues suggests that any local carcinogenic factors are not inducing COX-2 protein, although

there is COX-2 mRNA expression in these specimens, presumeably due to the increased sensitivity of RT-PCR compared to immunohistochemistry.

What might the effects of myenteric COX-2 expression be in the inflamed bowel? For many years it has been appreciated that there is altered colonic motility in the inflamed colon[230]. Such alterations in colonic motor and myoelectric activities with resultant changes in transit may contribute to the pain and diarrhoea characteristic of an exacerbation of IBD[231]. The mechanism of this dysmotility is uncertain but altered function of myenteric nerves and smooth muscle cells has been shown[231].

Pro-inflammatory cytokines alter enteric nerve function which in the rat is thought to be mediated by PG production, primarily PGE_2[225], with increased synthesis of this prostanoid in the rat myenteric plexus. PGE_2 has also been shown to modulate both non-adrenergic and non-cholinergic(NANC) transmission in the rabbit intestine[232], and to stimulate acetyl choline release from guinea pig myenteric plexus neurons with resultant changes in colonic contractility suggesting an effect via neural mechanisms[233].

However, the effects of such prostanoids on colonic motility in vivo are less certain. A decrease in sigmoid contractility occurs in humans during an intravenous infusion of PGE_2[234], whereas stimulation of the distal colon occurs in the rabbit after a similar infusion[235], suggesting that the effects of prostaglandins on the control of colonic motility may be species specific.

Prostaglandin receptors have been reported on both enteric neural and smooth muscle cells of the guinea pig ileum[236,237], but as yet no reports of similar receptor expression has been shown in man.

As already alluded to, in chronic IBD there are changes in motility and to note specifically in chronic UC there is reduced colonic contractility[238], a reduction in basal colonic intraluminal pressure[239], and a marked reduction in postprandial motility[240]. In

both human IBD and animal models of colitis there appears to be a relationship between altered colonic motility and abnormal local release of various inflammatory mediators of which prostaglandins such as PGE_2 are of considerable importance(241,242).

Toxic megacolon is a severe and life threatening consequence of the dysmotility of acute IBD. Initial work by Middleton et al(105) and more substantive evidence from Mourelle *et al* (106) have suggested a candidate inflammatory mediator for this toxic dilatation, that being $NO^{\cdot}$, as they have shown increased expression of iNOS in the muscle layers of the colon in this condition and have implicated $NO^{\cdot}$ production at this site as the cause of the reduced contractility and consequent dilated megacolon. None of the specimens that I used came from patients with toxic dilatation and therefore it is not surprising that there was an absence of iNOS expression in the muscle layers of these tissues(data not shown). The possible role of COX-2 production at this site is currently unknown, but with COX-2 expression in both smooth muscle and neural cells in the inflamed colon, it is plausible that the resultant prostanoids from COX-2 enzyme activity at this site might be of importance in the pathogenesis of this marked dysmotility. Therefore, my data suggests a new finding of COX-2 expression in neural cells of the myenteric plexus in the inflamed colon, and suggests that such enzyme expression at this site may link colonic inflammation, prostaglandins and dysmotility.

It is interesting that in the non-inflamed colon there is an absence of COX-2 protein production, unlike the case for iNOS which my data shows is expressed in the non-inflamed mucosa of the human colon. It is suggested that these two enzyme systems are induced by similar stimulants such as cytokines and LPS (215-217), and that they are co-inducible(220), and that $NO^{\cdot}$ might modulate COX-2 expression and/or activation(222,223). The fact that I have found iNOS in the normal colonic mucosa with an absence of COX-2 suggests that the stimulus to iNOS production does not stimulate COX-2 protein

production, and that the resultant NO· produced at this site cannot be stimulating mucosal COX-2 expression, but theoretically could be down regulating COX-2 protein expression. Further experiments are required to investigate these possible mechanisms of interaction in the normal colon.

In the same way considering the inflamed specimens, although my data is consistent with previous reports suggesting mucosal epithelial and inflammatory cell COX-2 expression in IBD it also suggests a novel site of this enzyme production, i.e. the myenteric plexus. As myenteric neural iNOS was not detected, it could be said again that the stimulus to COX-2 production in neural and smooth muscle cells of the myenteric plexus is different to that which is causing co-expression of iNOS and COX-2 in the mucosa. However it has been shown that iNOS expression does occur in the muscle layers of the colon in toxic dilatation but as mentioned earlier none of the patients in my experiments had toxic dilatation prior to colectomy.

The possible effects of mucosal COX-2 production in IBD include pro-inflammatory effects such as inducing epithelial chloride secretion(243) and resultant diarrhoea, and through the vasodilatory properties of the prostanoids mucosal hyperaemia. PGs also participate in regulation of cytokine synthesis by mononuclear cells(244), with possible down regulatory effects on the inflammatory cascade.

Further anti-inflammatory effects of prostaglandins which may be COX-2 derived include the ability to reduce:

1.The severity of colitis in a TNBS model and in other models of colitis(245-247).

2.The production of reactive oxygen metabolites(248).

3.The production of a number of inflammatory mediators contributing to the pathogenesis of human IBD and experimental colitis, including leukotriene B_4 and TNFα(249).

Prostaglandins can also reduce leukocyte adherence to the vascular endothelium, a feature that is demonstrated by the effect of NSAID (non-steroidal anti-inflammatory drugs) on increasing such adhesive interactions as they act by inhibiting prostaglandin synthesis(250). It can be seen, that the resultant PG synthesis from COX-2 induction in IBD, may exert many anti-inflammatory actions on the bowel, which in many ways is very similar to the scenario of $NO^{\cdot}$ production via iNOS induction in this condition.

It has been appreciated for many years that NSAIDs can exacerbate IBD(251). The mechanism of such exacerbations is not completely understood but it is believed to be via COX inhibition, with the earlier NSAIDs having greater selectivity for COX-1 over COX-2 suggesting a major contribution of COX-1 derived PGs to maintaining mucosal integrity in the colon. Indeed there have been suggestions that the anti-inflammatory effects of NSAIDs relate to COX-2 inhibition, and that the gastrointestinal injury, particularly the gastropathy, induced by these drugs relates to COX-1 inhibition(205). NSAIDs selective for COX-2 inhibition are now available and are shown to be effective in reducing inflammation whilst being relatively safe to the gastrointestinal mucosa. The contribution of PGs produced by COX-2 in the setting of colonic inflammation has been clearly demonstrated in a TNBS rat model of colitis(218). The severity and indeed lethality of the colitis was related to the degree of COX-2 selectivity of NSAIDs fed to the rats, with the more highly selective COX-2 inhibitors causing in many cases colonic perforation and death. The explanation being that COX-2 derived PGs are protective or anti-inflammatory in this animal model of colitis. It remains to be established whether the more selective COX-2 inhibitors have the same effects in human IBD, but there remains the concern that in the presence of colonic COX-2 expression inhibition of this enzyme may be deleterious to IBD. Of course in the normal setting with an absence of

COX-2 protein expression in the colon, then as analgesics such selective drugs may prove useful and sparing to the gastrointestinal tract.

Ulcerative colitis and to a lesser extent, Crohns colitis are associated with an increased risk of colorectal cancer. COX-2 is expressed in colonic adenomas and in adenocarcinomas in man[227-229]. Mutations of the FAP(familial adenomatous polyposis) gene are present in colorectal carcinomas. Recent work by Oshima *et al*[252] has shown the importance of COX-2 expression in polyp formation. Using APC gene knockout mice(model for human familial adenomatous polyposis), which have large numbers of COX-2 expressing polyps, when these mice were crossed with COX-2 knockouts there was a marked reduction in the formation of colonic polyps in the colons of these animals. This suggests that PGs produced through COX-2 may play a role in the increased risk of colonic cancer in IBD, again in a similar manner to which increased $NO^{\cdot}$ production via increased nitrosamines, direct mutagenesis or the production of peroxynitrite with all its carcinogenic potential may explain the increased incidence of colorectal cancer in IBD. Hence, the dual induction of both these enzymes by inflammation may have a fascilitatory effect on perpetuating inflammation and promoting carcinogenesis. COX-2 inhibition seems an attractive means of reducing cancer in IBD but with the reservations discussed above. However in high risk families or those with previous tumours in the absence of colonic inflammation, the use of selective inhibitors may be even more beneficial than conventional NSAIDs and aspirin in reducing the occurrence or progression of colorectal cancer and polyps[253-256].

Therefore, in summary my data confirms epithelial COX-2 expression in active IBD but extends the sites of localisation in man, to include neural and smooth muscle cells of the myenteric plexus. Such enzyme expression at this submucosal site, with the known

effects of PGs on colonic motility, could be a link between inflammation, PGs and colonic dysmotility that is seen in active IBD.

Chapter 6- Final conclusions and summary

Initial colonocyte cell experiments suggested that LPS alone was enough of a stimulus to induce iNOS. My initial hypothesis was that epithelial colonocytes are continuously in contact with a changing luminal environment. It was postulated that in the normal human colonocyte there may be an adequate stimulus to iNOS expression in the absence of inflammation, such as occurs in conditions such as IBD. Using the techniques described, I have presented my findings which suggest that iNOS is expressed in the non-inflamed bowel, being localised to the surface epithelium and conclude that there is some luminal stimulus as yet undetermined producing this enzyme expression. This observation of 'constitutive' iNOS expression is not entirely novel, but to date only one other piece of research has shown such a phenomenon and in a fewer number of patients, being somewhat limited by using the technique of immunohistochemistry, without illustrating the specificty of the primary iNOS antibody used in human colon.

I have disscused the possible effect of local cytokine production(in the normal surgical specimens who all had colorectal cancer) and the effects of surgical manipulation and have illustrated the production of iNOS protein in completely normal colons, and indeed in unprepared normal colons with similar cellular expression, to their surgical counterparts.

Furthermore, although it is postulated with a good deal of supporting data that iNOS expression is involved in the pathogenesis of IBD, in the normal colon, epithelial iNOS is not associated with tissue damage. This suggests that iNOS/NO· production is not deleterious to the colonocyte in this setting, although cellular toxicity can occur through means other than macroscopically evident necrosis.

The possible role of iNOS in the normal colon has been discussed and includes beneficial effects such as creating an oxidative barrier to micro-organisms and thereby

limiting invasion and translocation, and maintaining mucosal blood flow and integrity. Of course the actions of iNOS in the absence of other inflammatory mediators may in no way resemble its effects in the setting of mucosal inflammation as occurs in IBD, when there is the production of peroxynitrite and other reactive oxidative products of NO· which will perpetuate the inflammatory state, with likely tissue damage. Again, NO· may not be all toxic in IBD but might exert anti-inflammatory effects such as downregulation of certain pro-inflammtory mediators and cytokines such as NF-κB, and TNF-α, along with inhibition of leukocyte-endothelial interactions via downregulation of adhesion molecules. Therefore it is by no means certain what role iNOS/NO· is playing in IBD, i.e. 'friend or foe'.

The other possible effects of epithelial NO· is one of protection against or production of a carcinogenic state. No data is available, but iNOS/NO· in the normal colon may induce colonocyte apoptosis thereby removing cells with DNA abnormalities etc, which if unchecked could develop into a malignant clone. However, it may be that this NO· is infact protecting the colonocyte against apoptosis and along with the ability of NO· to increase local nitrosamine concentration, directly deaminate DNA, cause DNA strand breaks and lipid peroxidation it could be suggested that NO· is far more likely to have carcinogenic potential as opposed to antineoplastic effects. As mentioned it is not known whether iNOS production causes or protects against apoptosis in the normal colon, but either is a theoretical possibilty.

Real advances in understanding the role of increased iNOS/NO· in IBD and its possible role in the normal colon would occur if there were selective inhibitors of inducible nitric oxide synthase available for human useage. To date, there are no known such inhibitors and therefore all suggestions are limited by being theoretical only.

Due to a wealth of data suggesting interaction between iNOS and another inducible enzyme-COX-2, I examined the expression of this isoform in both IBD and the normal colon. My observations include that COX-2 protein is not expressed in the normal colon, a fundamental difference from iNOS. From these observations it is postulated that the stimulus to iNOS in the normal colonocyte is unable to induce COX-2 in the same manner. This in itself is interesting and suggests that in the absence of inflammation the mechanisms of induction of these two enzyme systems are fundamentally different, and indeed may be in some way differ in the inflammatory state also. I have shown COX-2 expression in the inflamed epithelium of some of the IBD specimens in line with previous studies, but have also shown strong expression in neural cells of the myenteric plexus, as yet an unpublished finding.

In comparison, iNOS is only produced in the muscle layers when there is marked, toxic dilatation. As noted from my experiments, iNOS is not localised to such sites in the inflamed tissues, noting that all specimens evaluated were from patients without toxic dilatation. It is suggested that in colonic toxic dilatation that occurs in IBD that $NO^{\cdot}$ is the mediator of this dilatation. I have shown that COX-2 is expressed in the neuromuscular layers in the inflamed colon before dilatation has occurred and suggest that local PGs released at this site provoke in part, the dysmotiltiy of this condition.

I have illustrated a fundamental difference in iNOS and COX-2 expression as although they are coexpressed in the colonic epithelium in IBD, suggesting a common stimulus,the situation is different in the sub-epithelium with COX-2 expressed at this site in the presence of inflammation, at an earlier stage to iNOS. This reflects differences in either the type or sensitivity to the stimulus for induction at this site ,or that COX-2 is expressed more easily than iNOS in these cells.

Unlike the case for iNOS, there are selective inhibitors of COX-2 available for human use. Utilising the selectivity of these drugs has shown that PGs derived from COX-2 activation in animal models of colitis are beneficial, suggesting an anti-inflammatory role for COX-2 in IBD. This again is in contrast to the situation when selective iNOS inhibitors are used in animal models of colitis where disordant results have been obtained.

Therefore it may be that mucosal COX-2 is protective in IBD, however the same may not be for COX-2 production in the myenteric plexus. Here it is conceivable that inhibition of COX-2 activity would improve the dysmotility of an exacerbation of colitis and might even prevent the state of toxic dilatation from occurring. There is currently too little data to advocate the use of selective COX-2 inhibitors during active IBD.

In summary, I have demonstrated the constitutive expression of iNOS in the non-inflamed human colon and have suggested plausible effects of resultant epithelial $NO^{\cdot}$ production both beneficial and detrimental.

Although, I have demonstrated that iNOS and COX-2 are coexpressed in the epithelium in IBD, COX-2 is also produced in the myenteric plexus and maybe involved in the dysmotility of IBD. Selective COX-2 inhibition may improve motility but to the detriment of mucosal integrity.

Future work

The work performed in this thesis suggest that further studies are required to clarify certain points raised by the data. Firstly, to clarify the possible stimulus to iNOS production in the normal colonocyte in vivo. This might be achieved by excluding some dietary component such as red meat or the like from the diet or by the introduction of pre and/or pro-biotics to the diet thereby creating a controlled manipulation of the colonic flora and therefore the luminal microenvironment. Secondly, to evaluate the relationship between iNOS expression and apoptosis and microcellular damage in the normal colonic epithelium, using insitu techniques to clarify apoptosis in vivo in the tissue itself.

Thirdly, to evaluate in cell culture(e.g.HT-29) the interactions between iNOS and COX-2 under various stimulants with regards to enzyme expression and activation to try and determine what interactions are occurring specifically in colonocytes and any differences in colonocyte behaviour with these stimuli. Fourthly, if available, it would be useful to evaluate the effects of known stimulants to COX-2 expression in neural cells(i.e. in culture) to ascertain what is inducing COX-2 in the myenteric plexus of the inflamed bowel. Lastly, to investigate the effects of selective COX-2 inhibitors on colonic motility in a suitable animal model of colitis, thereby suggesting a possible role in human IBD depending upon the effects noted.

Captions to tables and legends

Figure 1. Immunohistochemistry of HT-29 colonocytes

Immunolocalisation of iNOS protein in stimulated HT-29 colonocytes is shown in figure 1c by the presence of brown staining within the cytoplasm of the cells indicating iNOS protein expression at this site. Figure 1a shows an absence of such immunolabelling when the iNOS antibody is applied to unstimulated colonocytes, and figure 1b shows an absence of staining when normal rabbit serum is applied to the stimulated colonocytes.

Figure 2. iNOS RT-PCR

Figure 2a is an example of qualitative RT-PCR analysis of iNOS mRNA expression in both stimualted colonocytes and surgical specimens showing the ubiquitous presence of a 424bp DNA product indicating iNOS mRNA.

MW=DNA size markers(indicating 400 bp left lane). Lane 1-stimulated colonocytes, lanes 2-7-normal surgical tissue, lane 8-negative control, and lanes 9&10-inflamed colonic mucosa.

Figure 2b is an example of kinetic PCR iNOS mRNA analysis in a diseased(left hand set) and a normal specimen(right hand set).It can be seen that there is increased product in the diseased sample, and indeed on densitometrical analysis comparing diseased with normals there were up to X6 iNOS mRNA product compared to the normals when standardised for G3PDH expression.

G3PDH-Glyceraldehyde 3 phosphate dehydrogenase.PCR cycle lengths:G3PDH-30-34, iNOS-41-45.

Figure 3. iNOS immunoblotting

Figure 3 shows immunoblotting of normal surgical mucosal specimens with the primary iNOS antibody showing a protein band of approximately 130 kilodaltons in normal mucosa consistent with full length iNOS protein expression. Lane 1- application of iNOS

antibody/peptide to normal mucosa showing an absence or neutralisation of the protein band. Lane 2-application of iNOS antibody alone to normal mucosa showing the presence of a protein band consistent with iNOS expression. Lane S-application of the iNOS antibody to the iNOS recombinant protein standard(smaller sized bands indicating iNOS degradation on storage).

Figure 4 iNOS immunohistochemistry

Immunolocalisation of iNOS in surgical specimens using standard ABC vectastain technique. Figure 4a is a H&E stain of a section of normal colonic mucosa,showing an absence of inflammation. 4b shows the result of application of non-immune serum to such a section with an absence of immunostaining(lack of brown staining), acting as a control. Figure 4c however, is an example of positive iNOS localisation in the surface epithelium of normal mucosa when the primary antibody is applied to such a section. Figure 4d shows the same tissue after the application of the iNOS antibody which has been preincubated with the iNOS peptide. Here the immunostaining seen in fig 4c has been quenched, indicating the specificity of the positive iNOS immunostaining demonstrated in 4c.

Figure 4e is a H&E section of inflamed tissue showing mucosal inflammation and destruction.

Finally figure 4f is an example of iNOS immunostaining in such a specimen showing mucosal labelling of iNOS protein but also inflammatory cells of the lamina propria(low and high power views shown).

Figure 5 COX-2 RT-PCR

Figure 5a-an example of qualitative COX-2 RT-PCR in surgical tissue demonstrating the presence of a 484 bp cDNA product consistent with COX-2 mRNA in all specimens

analysed. Lane 1,3-6-normal tissue, lane 2-negative control(absence of PCR primers) and lanes 7-10-inflamed tissue.

Figure 5b is an example of kinetic PCR analysis of COX-2 mRNA expression in a normal(1), and a diseased specimen(2). Densitometrical analysis revealed greater expression(up toX8) of COX-2 product in the diseased tissue compared to the normal when standardised for G3PDH expression. PCR cycle lengths:G3PDH-30-34, COX-2-31-35. M=DNA size markers(Gibco BRL,UK).

Figure 6 COX-2 in situ hybridisation

Figure 6a demonstrates an absence of COX-2 mRNA labelling when the antisense probe is applied to a non-inflamed specimen. Also there is no labelling apparent when the sense probe is applied to a diseased specimen. However, when the antisense probe is apllied to this diseased sample there is intense labelling of both neural and smooth muscle cells of the myenteric plexus, indicating the presence of COX-2 mRNA at these sites(fig 6b). MP-myenteric plexus, NC-neural cell, SMC-smooth muscle cells.

Figure 7 COX-2 immunohistochemistry

Figure 7a is a H&E stain of the myenteric plexus in the human colon. Figure 7b demonstrates the absence of immunolabelling(brown staining) when non-immune serum is applied to a diseased specimen. Figure 7c(i&ii) illustrates myenteric labelling(primarily neural cells) when the primary COX-2 antibody is applied to this diseased specimen. In converse to this, when this antibody is applied to normal tissue there is no immunolabelling seen as shown in figure 7d. Figure 7e depicts surface epithelial labelling in a diseased specimen, the other site of COX-2 staining seen in the colitic specimens, whereas figure 7e demonstrates an absence of such COX-2 immunolabelling in normal mucosa.

MP-myenteric plexus, NC-neural cell, SMC-smooth muscle cells.

Publications from thesis

1 Roberts PJ, Riley G, Morgan K, *et al.* The physiological expression of inducible nitric oxide synthase(iNOS) in the normal human colon.*J Clin Path*(in press).

2 Roberts PJ, Morgan K, Miller RM, *et al.* Neuronal expression of COX-2 in active inflammatory bowel disease. *Gut*(in press)

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