Analysis of Apoptosis Levels in Normal, Inflamed and Neoplastic Colonic Mucosa; The Effects of Non-Steroidal Anti-Inflammatory Drugs and 5-Aminosalicylic Acid on Human Colonic Epithelial Cell Viability

Thesis Submitted for the Degree of Doctor of Philosophy at the University of Leicester

by

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September 2003
Abstract

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Analysis of Apoptosis Levels in Normal, Inflamed and Neoplastic Colonic Mucosa; The Effects of Non-Steroidal Anti-Inflammatory Drugs and 5-Aminosalicylic acid on Human Colonic Epithelial Cell Viability

Sonya G Jackson  September 2003

Abstract

Numerous epidemiological, in vivo and in vitro studies have demonstrated the chemopreventive potential of NSAIDs, and it is thought that this chemoprevention may be due to the induction of apoptosis within malignant cells. The 5-aminosalicylic acid (5-ASA) compounds are structurally similar to the NSAID aspirin, and have also been shown to reduce tumour growth in recent studies.

There are a number of mechanisms by which this chemoprevention may occur, including inhibition of cyclooxygenase expression, induction of apoptosis through alterations in expression of the Bcl-2 family members, and activation of the peroxisome proliferator activated receptor family of nuclear hormone receptors.

The studies presented in this thesis were designed firstly, to analyse cell turnover in normal, inflamed and neoplastic colonic mucosa, and secondly to compare and contrast the effects of the NSAID indomethacin, and 5-ASA on human colonic adenocarcinoma cell lines, and to gain further information regarding the mechanistic pathways underlying the chemopreventive properties of these compounds.

Cell turnover was analysed in colonic mucosa using immunohistochemistry. The effects of indomethacin or 5-ASA treatment on cell viability, apoptosis and cell cycle distribution in human colonic cell lines was analysed by immunohistochemistry and flow cytometry. Changes in Bax, Bcl-XL and PPAR expression were analysed by RT-PCR.

Indomethacin reduced cell viability, increased apoptosis, and caused cell cycle accumulation in the G0/G1 phase of the cell cycle. These effects were not dependant on cyclooxygenase expression. No changes to either cell viability or apoptosis levels were seen in cell lines following 5-ASA treatment. PPARγ, and both PPARα and γ expression increased following treatment of cell lines with 5-ASA or indomethacin respectively.

In conclusion, these studies suggest that 5-ASA and indomethacin may have a different mode of action with regards to chemoprevention. Both 5-ASA and indomethacin may result in activation of the PPAR nuclear receptors, this presents an alternative mechanistic pathway for the observed chemopreventive properties of these compounds.
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Abbreviations

5-ASA  5-aminosalicylic acid
AP    alkaline phosphatase
BCIP  5-bromo-4-chloro-3-indolyl phosphate
BSA   bovine serum albumin
CD    Crohn's disease
CDNA  copy deoxyribonucleic acid
Cox   cyclooxygenase
DICD  detachment-induced cell death
DAB   3,3-diaminobenzidine
DNA   deoxyribonucleic acid
DMSO  dimethyl sulfoxide
DMEM  dulbecco’s modified eagle medium
dNTP  deoxynucleoside triphosphate
EDTA  ethylenediaminetetraacetic acid
FCS   foetal calf serum
FAP   familial adenomatous polyposis
FADD  fas-associated death domain protein
FACS  Fluorescence-activated cell sorter
GAPDH glyceraldehyde-3-phosphate dehydrogenase
HNPCC hereditary non-polyposis colorectal cancer
HBSS  Hank’s balanced salt solution
HPLC  high performance liquid chromatography
5-HSA  2,5-dihydroxybenzoic acid
IBD   inflammatory bowel disease
NEAA  non-essential amino acids
PI    propidium iodide
HRP   horseradish peroxidase
PBS   phosphate buffered saline
PP    peroxisome proliferator
PPAR  peroxisome proliferator activated receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>PMN</td>
<td>polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>PPRE</td>
<td>peroxisome proliferator response element</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MMP</td>
<td>microsatellite mutator phenotype</td>
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<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
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<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>UPH₂O</td>
<td>ultra pure water</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
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Chapter 1.

General Introduction.
1. Introduction

The chemopreventive ability of NSAIDs, first described by Waddell and Loughry in 1983, is now a generally accepted phenomenon. However, despite extensive studies, the pathways through which this chemopreventive effect occurs remains unknown. More recently, the 5-aminosalicylic acid (5-ASA) compounds, have also come under the spotlight in the search for chemopreventive agents. The 5-ASA containing compounds are commonly used in the treatment of the idiopathic inflammatory bowel disorder, ulcerative colitis (UC), a disease known to predispose to colonic cancer. The mechanisms through which 5-ASA acts to bring about remission of UC is unknown; and whether or not 5-ASA acts as an anti-inflammatory agent through the same pathways as the NSAIDs (i.e. through inhibition of cyclooxygenase) is unclear.

As colorectal cancer results in an abnormal accumulation of cell number, it is clear that deregulation of normal cell growth/death mechanisms may play an important role in neoplastic progression. Abnormal cell turnover may also play a vital role in the pathogenesis of other diseases within the colon, including UC. The aim of studies presented in chapter three of this thesis, were to understand the normal mechanisms of cell turnover within the colon, and to examine how changes in cell turnover may be involved in inflammatory or neoplastic colonic disorders.

NSAIDs have been shown to induce apoptosis of malignant colonic cells. Studies presented in this thesis were designed to analyse the chemopreventive potential of NSAIDs, and to compare this with chemopreventive potential of the compound 5-ASA. The pathways by
which observed chemoprevention may occur were examined, with regards to changes in the expression of genes involved in apoptosis. Recently the peroxisome proliferator activated receptor (PPAR) group of nuclear hormone receptors have been shown to be possible targets of NSAIDs; this, coupled with recent evidence of possible PPAR involvement in the regulation of cell differentiation, apoptosis, and neoplastic development, puts forward the PPARs as new target in the search for pathways activated during NSAID chemoprevention.

This introductory chapter provides the background to the studies presented in this thesis. The normal, inflamed and neoplastic colon is described; and the importance of the apoptotic process, alongside the role of various genes, which are of particular interest in these studies, is presented. The Cox enzymes (Cox-1 and Cox-2) are important in inflammatory processes within the colon, and Cox-2 has been shown to be up regulated in colonic carcinoma. Whilst NSAIDs reduce inflammation by inhibition of Cox, it is not thought that the inhibition of Cox alone is necessary or sufficient for chemoprevention; the evidence for Cox independent pathways is discussed, along with possible alternative pathways that may be activated, in particular with regards to activation of the PPARs. Finally, the evidence for chemoprevention by 5-ASA compounds is introduced.
1.1 The Colon – Normal Histology

The principle functions of the colon are the absorption of fluid and electrolytes from the ileal effluent and the formation and propulsion of faeces towards the rectum. Consequently the colon itself consists of a thick muscular wall, capable of powerful contractions, and a surface mucosal layer, which is lined with columnar absorptive and goblet secreting epithelial cells. These epithelial cells make up a series of crypts, which extend down to the muscularis mucosa; the result of this architecture is a greatly increased functional surface area. The crypts are positioned perpendicular to the muscularis mucosa, they maintain a regular architecture and are not branched within healthy individuals (figure 1.1). At the crypt base, undifferentiated stem cells are found, these cells will migrate up the crypt wall to the surface, during this process they will differentiate into columnar absorptive, or goblet cells. Eventually, these cells will be shed at the lumen; this entire process, from undifferentiated stem cell, to loss at the luminal surface, takes between three and eight days. The mucus-secreting goblet cells predominate at the base of the crypts, thin strips of muscularis mucosa extend up between the crypts and facilitate the passage of the mucus up the crypts where it is important in aiding the passage of luminal contents, and preventing trauma to the mucosa. The luminal surface, in contrast, is lined almost exclusively by absorptive, columnar epithelial cells, which are responsible for the transportation of water and ions.

Crypts are separated from each other by the lamina propria, which consists of connective tissues, capillaries, fibroblasts, inflammatory cells and lymphatic vessels, into which water is absorbed by passive diffusion. The majority of cells within the lamina propria are involved in forming an immunological response to potentially damaging agents that may be present within
the colon. Numerous leucocytes, including lymphocytes, are found scattered throughout the 
lamina propria, or organised into large aggregations known as lymphoid follicles (figure 1.2).
These lymphoid follicles can extend through the lamina propria into the submucosa and may 
cause a disruption to the normal architecture of the surrounding crypts. As well as being 
present within the lamina propria, lymphocytes can invade the colonic epithelium (Bartnik et 

1.2 Colonic Neoplasia

1.2.1 Colorectal Tumours. Colorectal cancer is an extremely common disease in the western 
world, and is the second leading cause of cancer deaths (Stevens and Lowe, 1995). By the age 
of 70, approximately 50% of the western population will have developed some form of a 
colonic tumour, ranging from benign polyps, to invasive carcinomas (Fahy et al, 1998). 

Hyperplastic polyps are sessile lesions commonly found amongst the older population. These 
polyps are generally less than 5 mm wide, and are mainly located in the sigmoid colon and 
rectum (DiSario et al, 1991). They may arise as a result of abnormal mucosal maturation or 
arquitecture, or may be a result of inflammation. Whilst they are generally believed to be non-
neoplastic, (Rex et al, 1992), the presence of hyperplastic polyps is associated with adenomas 
and carcinoma (Ansher et al, 1989; Blue et al, 1991 and Clark et al, 1985). This however 
could be due to genetic susceptibility of individuals to colonic tumours, or exposure of 
individuals to agents that promote tumour development, rather than direct development of 
neoplastic adenomas from hyperplastic polyps.
Figure 1.1 Normal colonic mucosa.

The section is stained with haematoxylin and eosin and orientated with the luminal surface (L) at the top of the figure. Crypts (C) are seen lined in parallel within the mucosa, and are separated by the lamina propria (arrows).
Figure 1.2 Haematoxylin and eosin stained section of normal colonic mucosa.

A lymphoid follicle (L) can be seen confined within the colonic mucosa (x16).
Adenomas are neoplastic polyps that arise mainly from proliferating dysplastic epithelia. Whilst adenomas are benign, they have been shown to have malignant potential (Williams et al, 1990) and are therefore clinically important. Adenomas can be classified into 3 different types, based on their morphological characteristics, these are tubular, villous and tubulovillous. Tubular adenomas are the most common adenomas found, they are generally small and grow on a stalk (pedunculated), villous adenomas are often larger and sessile. Tubulovillous adenomas, as the name suggests, show a combined tubular and villous morphology. Adenomas are of particular interest as the pattern of normal mucosa, to adenoma, to carcinoma is probably the pathway by which the majority of colonic carcinomas arise. The malignant potential of adenomas is dependant on size, type and level of dysplasia. Small tubular adenomas have low malignant potential, whilst villous adenomas greater than 4 cm have a malignant potential of around 40%. Risk of malignancy in tubulovillous adenomas depends on the proportion of villous morphology (O’Brien et al, 1990).

**1.2.2 Colonic Carcinoma.** Approximately 10% of benign colonic adenomas will develop into invasive adenocarcinomas (Fahy et al, 1998). Colonic carcinoma is generally a disease of the elderly population and has equal precedence between males and females, it has a peak incidence in the seventh decade of life, and is rare under the age of 40. If seen in younger patients, familial adenomatous polyposis (FAP) or inflammatory bowel disease is suspected as the predisposing cause. Although colonic carcinoma develops from certain inherited disorders, such as FAP and hereditary non-polyposis colorectal cancer (HNPCC), these conditions account for only 10-15% of cases. The remaining cases are sporadic and the etiology remains unknown, although colonic cancer risk is associated with family history,
diet, smoking, and alcohol consumption (reviewed by Potter, 1999). A marked geographical variation in the rates of colorectal tumours has been noticed, with western populations demonstrating increased incidence in comparison to countries such as Africa, South America, Japan and India. Diet has been implicated as the one of the main causes of these variations. The typical western diet, being high in carbohydrate and fat and low in dietary fibre may result in an increased transit time of faeces, this could result in increased exposure of colonic bacteria to luminal contents, allowing time for them to degrade substance such as bile acids, which can result in the formation of carcinogenic agents.

As mentioned, the presence of pre-cancerous lesions, such as adenomas is also an important factor in the development of carcinoma. In addition to this, long-standing inflammatory bowel disease (IBD) (see section 1.3) is a risk factor, especially if IBD has been present for more than 10 years, and involves the entire colon. The cumulative risk of carcinoma is thought to be about 1-3% if IBD has been present for 10 years, rising to 10% after 20 years duration (Kornfeld et al, 1997).

The development of colorectal cancer is a multistep process involving the inactivation of tumour suppressor genes and the activation of oncogenes. Various genes have been noted to be frequently altered in colorectal cancer; these include ras gene mutations, which are found in approximately 50% of human colorectal carcinomas, and deletions on chromosomes 5q, 17p and 18q (reviewed by Fahy and Bold, 1998). The tumour suppressor gene p53 is located on the short arm of chromosome 17 and is the most common genetic alteration found in colorectal cancer, occurring in more than 75% of cases. P53 mutations however, are rarely
seen in adenomas (Vogelstein et al, 1988). Approximately 75% of carcinomas and 47% of advanced adenomas have deletions on chromosome 18q, affecting a gene known as DCC (deleted in colorectal carcinomas). There is some debate as to whether DCC is indeed a tumour suppressor gene as experiments have failed to find an increase in tumour formation in heterozygous DCC<sup>+/−</sup> mice, furthermore loss of DCC function did not affect epithelial cell proliferation or differentiation within the colon (Fazeli et al, 1997). These observations however do not rule out a possible role of DCC in the pathogenesis of human colorectal cancer. Finally, the tumour suppressor gene Smad4 which, like DCC, is located on chromosome 18q, has been found to be frequently mutated in late stage colorectal carcinoma, and is associated with an invasive and metastatic phenotype (reviewed by Miyaki and Kuroki, 2003). Furthermore, mutations in Smad4 are thought to be responsible for the development of familial juvenile polyposis, which predisposes to the development of colonic cancer.

These four mutations, commonly found in colorectal cancer, help to build up a stepwise model for the genetic alterations that may be involved in tumour progression (figure 1.3), however it is important to realise that this by no means represents an exhaustive, or necessary model for mutations involved in colorectal neoplasia.

Familial adenomatous polyposis (FAP) is a condition resulting in the formation of hundreds of colonic adenomas and leading on to the development of carcinoma by the fifth decade of life (highlighting the importance of the adenoma – carcinoma sequence). It is a rare, autosomal dominant syndrome, affecting approximately 1:10000 people a year, and results from an inherited mutation in the APC. APC is located on chromosome 5q21 and mutations may be
present in different areas of the APC gene, however the majority of them lead to the formation of a stop codon, resulting in the production of a truncated protein. The APC protein is involved in reducing the levels of a cytoplasmic protein known as β-catenin. APC can bind to, and degrade β-catenin. If APC is mutated then it can no longer degrade β-catenin, and free β-catenin can move into the nucleus where it forms a complex with other proteins and induces the expression of genes involved in promoting cellular growth and differentiation.

Hereditary non-polyposis colorectal cancer (HNPCC) is the second main cause of familial colorectal cancer and is responsible for approximately 2-4% of cases (Lynch et al, 1996). It is an autosomal dominant condition resulting from mutations in the DNA mismatch repair (MMR) genes, the main consequence of which is loss of proofreading ability and inability to correct small insertions and deletions. In addition, colorectal cell lines carrying MMR mutations have been shown to be prone to further mutations and deletions. HNPCC results in the development of generally right sided, poorly differentiated colonic tumours, usually in the 5th decade of life.

It has been seen that the development of colorectal cancer can result from various mutations, which collectively lead to the loss of normal cell growth and death control. In addition to the genetic factors, environmental factors play a part, suggesting that the development of colorectal cancer is a complex balance between environmental exposure, individual susceptibility, and genetic mutation.
Figure 1.3 Genetic mutations involved in the stepwise progression of colonic cancer.

Colonic carcinoma is thought to arise from the progression of normal mucosa to adenoma to carcinoma, and the involvement of APC, Ki-Ras, DCC and p53 at various stages of this process is well documented. Mutations of APC are an early event in tumourogenesis, Ki-Ras has been shown to be mutated in approximately 50% of colonic cancers, DCC mutations are found in 47% of advanced adenomas and 75% of carcinomas, and p53 mutations, whilst rarely seen in adenomas are present in over 75% of colonic carcinomas. Adapted from Underwood, 1996.
**1.3 Inflammatory Bowel Disease**

**1.3.1 Idiopathic Inflammatory Bowel Disease.** There are two main types of idiopathic inflammatory bowel disease, these being ulcerative colitis (UC) and Crohn's disease (CD). Inflammatory bowel disease can present at any stage in life but has a peak onset between 15 and 25 years of age, and appears to affect males and females equally.

The aetiology of IBD is unknown but a geographical variation is seen, with high incidence in northern Europe and North America, and low rates in southern Europe, Asia, Africa and South America (Moses et al, 1998). In addition, certain populations have higher incidence, for example, both UC and CD are more common in white, than coloured people, and the incidence is higher in Jews than non-Jews (Yang et al, 1993). It is believed that there may be a genetic factor involved, as the risk of developing UC or CD is increased amongst first-degree relatives (Orholm et al, 1999). Whilst it is as yet unknown what genetic factors may predispose to IBD, recent studies have focused on the role of human leukocyte antigen (HLA) as a possible candidate. However many other genes have also been implicated, in both UC and CD. A susceptibility locus for CD has been mapped to chromosome 16, and mutations in the gene NOD2 which is located on the chromosome, have been implicated in the development of CD (Hugot et al 2001; Ogura et al, 2001). NOD2 has structural homology to the Apaf-1/CED-4 superfamily which are involved in the regulation of apoptosis, and has been shown to activate the nuclear factor NF-kB. It is thought that NOD2 plays a role in the recognition of bacterial lipopolysaccharides, and that mutations within this gene may lead to inappropriate inflammatory or apoptotic response following exposure to various bacterial pathogens.
In addition to genetic factors, certain environmental factors have been shown to be associated with IBD. Of these, smoking is the most studied, and has been shown to have opposite effects in UC and CD. Whilst smoking is known to decrease the risk of UC (Vessey et al, 1986, Boyko et al, 1987), it has been shown to increase the risk of CD (Tobin et al, 1987). The underlying reason for the protection against UC is unknown, although it may be related to altered colonic mucus production, reduced rectal blood flow, alterations in the proportions of helper and suppressor T-cells, or changes in cytokine production. Studies have been undertaken investigating the use of oral contraceptives and IBD. An increased risk was found in one study (Vessey et al, 1986), however this was not statistically significant. The effect of diet on IBD is still unclear, and requires further research. It is not yet known if UC and CD are two completely distinct diseases, or if they result from different reactions to the same aetiological agent. Both diseases are chronic, relapsing inflammatory disorders.

1.3.2 Ulcerative Colitis. UC can present at any age but has a peak incidence in people aged between 15 and 30 (Calkins et al, 1984, Andres and Friedman, 1999). The disease affects the colonic mucosa, resulting in areas of inflammation, mucosal damage and ulceration. Although the disease primarily affects the colonic mucosa, in fulminant disease the submucosa and muscle wall become involved. This can result in toxic dilation, which is potentially lethal if not treated surgically. UC begins at the rectum and can extend proximally and continuously for variable distances into the colon.
In the majority of patients, the disease is limited to the rectum, rectosigmoid or left colon at the point of diagnosis. 55% of patients present with proctitis, 30% with left sided colitis, and approximately 15% have pancolitis (Ghosh et al, 2000). The symptoms at presentation tend to correlate with disease severity, with patients with pancolitis generally having worse symptoms than those with proctitis only. Symptoms can include diarrhoea, often containing blood and mucous; and colonic pain, generally left sided and associated with defecation. In severe disease, patients may also present with fever, tachycardia, anemia, increased white blood count and colonic dilation.

Whilst the disease is characterised by periods of recurrence and remittance, some patients suffer from a continuous episode. In addition, between 5 and 13% of patients will suffer from a fulminating attack (McGee et al, 1992), when the entire thickness of the colon is affected, rather than the colonic mucosa alone. In fulminating UC, the colon becomes dilated, and the majority of mucosa is lost. In addition, extensive ulceration is seen and submucosa may be lost, underlying muscle becomes thin and can be exposed, the muscle fibres themselves often appear vacuolated and may be separated due to oedema. Fissuring ulceration may also be seen, splitting into the circular layers of the muscle wall. The onset of fulminating disease usually results in an emergency colectomy.

Microscopic features differ depending on whether the disease is active, fulminating or quiescent, and disease severity will be determined by the presence of certain histological features. In active UC, a diffuse inflammatory cell infiltrate is seen in the lamina propria, neutrophils invade the gland epithelium, and collect in the lumen of crypts, forming what are
known as crypt abscesses (figure 1.4). Goblet cell depletion is seen, crypts appear distorted, and lack of surface epithelium, indicating areas of ulceration may also be apparent. This ulceration is limited to the mucosa and lamina propria. Within the lamina propria, a diffuse infiltration of both plasma cells and lymphocytes are seen, eosinophils are also sometimes present. In fulminating UC, the inflammation extends through the mucosa, into the submucosa and muscle wall, and all types of inflammatory cells are present throughout the thickness of the colon. During quiescent UC, the colon may appear macroscopically normal, however, histological features of UC remain. Whilst the numbers of inflammatory cells decreases, and ulceration is no longer obvious, lymphoid follicles may remain, and crypts appear abnormal, shortened, branched and fewer in number.

As mentioned, the aetiology of UC is still unknown. Possible theories include bacterial or viral infection, environmental factors, genetic predisposition or an abnormal immunological host response to colonic infection. However extensive research into these areas has failed to provide any definite answers. An increased risk has been noticed however, amongst first-degree relatives of UC patients as mentioned previously.

An established risk of UC is the development of colorectal carcinoma. Two main factors that appear to be associated with the risk of developing carcinoma are the extent and duration of disease, with the greatest risk for patients with pancolitis for more than 10 years. Ekbom et al (1990) undertook a population based study and found a standard incidence ratio (ratio of observed colorectal cases/expected cases) of 1.7 for patients with proctitis only, 2.8 for those with left sided colitis, and 14.8 for patients with pancolitis. They also found an association
between age at diagnosis and risk of carcinoma. The overall risk of developing carcinoma within 35 years of diagnosis of pancolitis was found to be 30%, and 40% in patients who were diagnosed before the age of 15. The annual incidence of colorectal cancer in patients suffering from UC of greater than 10 years duration is between 0.8 and 1% (Andres and Friedman, 1999).

Patients suffering from UC are treated with various non-steroidal anti-inflammatory drugs (NSAIDs) containing 5-aminosalicylic acid (5-ASA). These include sulphasalazine, where 5-ASA is attached to sulphapyridine, this is then broken down by bacteria within the colon, releasing the active 5-ASA compound. Whilst treatment with this compound may result in remission in mild cases, its main function is to reduce the frequency of recurring attacks. The sulphapyridine carrier, however, results in side-effects within some patients, and therefore new carriers for 5-ASA are becoming more commonly used for treatment. These include mesalazine and olsalazine. Patients can also be treated with steroids; local rectal treatment with prednisolone or hydrocortisone is effective for the treatment of proctitis, and oral prednisolone or hydrocortisone is often administered in moderate and severe attacks. Even during quiescent disease, patient therapy with 5-ASA compounds is continued, meaning that exposure to this compound is long-term for UC sufferers.
**Figure 1.4 Severe ulcerative colitis.**

Haematoxylin and eosin stained tissue section, obtained from a case of severe ulcerative colitis. Crypt abscess (CA) were visible within the section. Large numbers of inflammatory cells can be seen throughout the lamina propria and there is extensive loss of surface epithelium.
1.3.3 Crohn's Disease. CD is a disorder affecting approximately 1 in 1500 people in the United Kingdom (Rampton, 1999). As with UC, it can affect people of any age, although peak incidences are seen in young people (15-30), and again in later life (60-80). Unlike UC, which affects males and females equally, CD has a greater incidence in females (Calkins et al, 1984), with reported ratios of between 1.1 and 1.8 (Andres and Friedman 1999). The aetiology is as yet unknown, although a genetic factor is likely to be involved as increased rates are seen amongst first-degree relatives of sufferers (Peeters et al, 1996). Whilst UC affects the colon only, CD can affect any part of the alimentary tract, although the small bowel is the most commonly affected area. The colon and small bowel are involved in about 30% of cases, and the colon only in a further 30%. Unlike UC, skip lesions, i.e. areas of normal bowel between diseased areas are seen. Patients generally present with diarrhoea, abdominal pains and weight loss.

Macroscopically, the mucosa takes on a cobblestone appearance; this is due to deep-fissured ulcers between folds of mucosa. CD is not confined to the colonic mucosa as in UC, and transmural inflammation is seen. This leads to thickening of the bowel wall due to oedema and fibrosis. Stricture formation may occur, which can result in bowel obstruction.

Histologically, inflammation is seen throughout the bowel wall, areas of inflamed serosa can become attached to similarly inflamed areas, or other areas within the abdomen. Fissures can be seen extending down through the bowel wall; and are often lined by inflammatory cells. The submucosa may be fibrotic and often appears widened due to oedema. Finally, crypt abscesses, although less frequent than in UC are also common in CD. Granulomas are a
histological feature seen in approximately 50-70% of cases (figure 1.5). Due to this granulomatous infiltrate observed in CD, the possibility of an infective cause seems appealing. Exposure to the measles virus has been implicated; Ekbom et al (1996) followed up four children born to mothers infected with measles during pregnancy, and found that three of the children went on to develop CD. However, conflicting data regarding measles exposure and CD has been published, and the role of this virus remains unclear. Whilst further mycobacterium DNA and virus particles have been found in some patients, results are again conflicting, and as yet no agent has a proven role in development of the disease.

As with UC, a long-term complication of CD is the development of carcinoma, both within the small and large intestine. Again, the risk is associated with extent of disease, being highest for patients with extensive colonic involvement, and in those diagnosed with CD before the age of 25. The cumulative risk of colorectal carcinoma is 8% after 22 years, representing an 18 fold increase compared to the general population (Andres et al, 1999). Whilst treatment in mild cases, is usually confined to controlling the symptoms of disease, such as diarrhoea, patients with more severe disease are frequently treated with anti-inflammatory and immunosuppressive drugs. Oral treatment with corticosteroids can be useful in treatment of patients with severe attacks. Various other immunosuppressive and anti-inflammatory treatments have been used, with varying success. Where there is colonic disease, patients are often given the NSAIDs mesalazine or sulphasalazine, in addition to rectal steroids.
Figure 1.5 Crohn's disease

Haematoxylin and eosin stained tissue section obtained from a case of Crohn's disease. Transmural inflammation with thickening of the colonic mucosa can be seen.
1.4 Apoptosis

1.4.1 Introduction. Two distinct forms of cell death are recognised, the first, termed necrosis, is a rapid process that results from a severe damaging stimuli to the cell. Following this injury, cytoplasmic swelling and nuclear condensation occurs, the cellular membrane ruptures, as do lysosomes within the cell, and finally, organelle membranes rupture and the nucleus disintegrates. This process results in an inflammatory response. Programmed cell death, otherwise known as apoptosis is a controlled form of cell death that is distinct from necrosis.

The structural features of apoptosis were first described by Kerr et al in 1972, and these features can be used to distinguish apoptotic cells from necrotic ones. Firstly, the nuclear material compacts and aggregates along the nuclear envelope, it was later shown that the DNA is degraded by an endonuclease that cleaves between nucleosomes, resulting in DNA fragments of 180-200 base pairs in length (Wyllie et al, 1980, Wyllie 1980). Concurrent with nuclear changes are changes within the cytoplasm. Whilst cellular organelles remain intact, the cell shrinks and separates from its neighbours. Phospholipids in the plasma membrane are flipped and the cell forms buds, which separate, forming apoptotic bodies. These apoptotic bodies are quickly phagocytosed and degraded by surrounding cells or macrophages. The fact that apoptotic cells form small vesicles, which are then engulfed by surrounding cells, means that leakage of cellular contents is prevented; in turn this also prevents an inflammatory response being activated. This controlled form of cell death therefore, results in minimal damage to surrounding tissues. In vitro however, apoptotic bodies are rarely engulfed by surrounding cells and instead enter a final degradation process, which has been termed secondary necrosis.
Apoptosis is a vital process, both during embryonic development (for example, in the removal of interdigital webs), and for the maintenance of tissue homeostasis within the adult. As mentioned, epithelial cell lining the gastrointestinal tract have a turnover rate of between three and eight days, and the balance of cell proliferation, must be matched with cell loss in order to maintain normal tissue morphology. Apoptosis is thought to play at least a partial role in the removal of differentiated cells at the luminal surface, this is backed up by the detection of apoptotic cells at the top of crypts (Benard et al, 1999; Moss et al, 1999) and the fact that rates of apoptosis within the colon balance the rates of proliferation (Hall et al, 1994). The role and control of apoptosis within the normal colon will be further discussed in section 1.4.5.1.

Apoptosis is an energy dependant process requiring the transcription or activation of intracellular proteins, and this in turn must be tightly controlled to ensure an appropriate cellular response. Apoptosis can be activated, carried out or inhibited by many different mechanisms (Huppertz et al, 1999).

Apoptosis may be induced by various factors, including intra-cellular pre-programming, exposure to mild cytotoxic agents such as radiation, exposure to mild heat treatment, lack of growth or survival factors, oxygen deficiency, and through extra-cellular signals such as hormones or ligand binding to death receptors (e.g. FAS/FAS ligand). Apoptosis can be broadly divided into initiation, signalling and execution stages.

Once apoptosis has been initiated various signals are activated within the cell. The mitochondrion is known to play a central role in the execution of apoptosis (Susin et al, 1998). Mitochondrial membrane permeability increases and there is a decrease in the mitochondrial
membrane potential, this is accompanied by oxidative stress, and the release of cytochrome c into the cytosol. Cytochrome c release may be blocked or induced by the Bcl-2 and Bax respectively (see sections 1.4.4 and 1.4.5).

The signalling phase is followed by the execution phase, during which caspases are known to play a key role. The execution phase results in the degradation of cellular components and chromatin. Finally the apoptotic bodies of the cell are taken up by macrophages.

1.4.2 Caspases. Genes that were involved in the control of programmed cell death were first discovered in the nematode *Caenorhabditis elegans*. A number of these cell death (ced) genes were discovered, ced-3 and ced-4 were required for cell death, whilst ced-9 had a protective effect. Ced-3 was cloned and was found to act as a cysteine protease (Yuan et al 1993). Multiple mammalian homologues of ced-3 have since been discovered, these proteases are collectively known as caspases (reviewed by Nunez et al, 1998). Caspases are synthesised as proenzymes, which become activated by proteolytic cleavage following the initiation of apoptosis. The activation of caspases is thought to play a pivotal role in the execution of apoptosis, and within mammals, the apoptotic process is largely suppressed by the presence of caspase inhibitors. However the role of individual caspases remains unclear, and various experiments suggest that caspases may be essential or redundant to the apoptotic process in a manner that is dependant on the cell type and apoptotic stimuli (Woo et al, 1998; Bergeron et al 1998; Hakem et al, 1998).

Broadly speaking, caspases can be divided into either initiator or execution caspases, depending on their role in the apoptosis cascade (Fraser and Evan, 1996). Initiator caspases
are activated by inducers of apoptosis, such as Fas-L binding to the Fas receptor. This results in the recruitment of adapter proteins, such as Fas-associated death domain protein (FADD), which leads to the formation of a multiprotein complex known as the death-inducing signalling complex (DISC). Interaction of FADD with pro-caspase-8 results in the proteolytic cleavage and therefore activation of this initiator caspase (Medema et al, 1997).

Following activation, caspase-8 has two main functions, the first is the activation of downstream caspase targets (Srinivasula et al, 1996) which then bring about the morphological changes that are associated with apoptosis (Martin et al, 1995), and the second is the activation of the pro-apoptotic protein Bid, which in turn triggers the release of cytochrome c from the mitochondria (Kuwana et al, 1998). Other initiator caspases can be activated through similar or distinct signalling pathways. Generally the activation of the initiator caspases leads to morphological changes within the cell, for example membrane blebbing, phosphatidylserine flip and cleavage of cytoskeletal proteins such as fondrin and actin (Cryns et al, 1996; Kayalar et al, 1996). Apoptosis is still reversible at this stage.

As mentioned, in addition to the activation of Bid, a function of caspase-8 is the activation of further caspases, these are the execution caspases, such as caspase-3, -6 and -7. Once the execution caspases are activated, the cell is committed to undergo apoptosis. The activation of execution caspases results in further cleavage of various structural and housekeeping genes, including cytokeratin 18, nuclear lamins A, B and C, PARP and topoisomerase II. Further targets include proteins normally involved in the synthesis and repair of DNA, such as the nuclear replication factor MCM3, the large subunit of DNA replication factor C and human Rad51 (Rhéaume et al, 1997; Schwab et al, 1998). In addition, caspase-3 has been shown to
activate the endonuclease DFF40/CAD by the cleavage of its inhibitor ICAD/DFF45 (Sakahira et al, 1998; Liu et al, 1997), which leads to the internucleosomal digestion of DNA, producing the characteristic “DNA ladder” seen when DNA from apoptotic cells is separated by electrophoresis. Further evidence for the role of this endonuclease in apoptosis comes from DFF/CAD mutated cells, which are protected from DNA degradation, but still undergo the other morphological events associated with apoptosis (Sakahira et al, 1998; Liu et al, 1998).

Caspase inhibitors have been shown to prevent apoptosis in normal intestinal cells within mice (Scheving et al, 1998), and the activation of caspase-3 during detachment induced cell death of normal human colonic cells has been demonstrated (Grossman et al, 1998). These experiments highlight the role of caspases during normal apoptosis of colonic epithelium.

An antibody has been developed that recognises the p20 subunit of the cleaved, and therefore activated, caspase-3 fragment. As the pro-form is not detected, this antibody is a useful marker for distinguishing between apoptotic and normal cells.
1.4.3 Bcl-2 Family.

1.4.3.1 Bcl-2. The apoptosis-suppressing Bcl-2 gene was first discovered at the break-point of the t(14;18) chromosomal translocation, which is associated with certain human B-cell lymphomas. The result of this translocation is increased Bcl-2 expression and indeed these lymphomas are attributed to cellular accumulation resulting from failure of cell death, rather than an increase in cellular proliferation.

The inhibition of cell death by Bcl-2 was first demonstrated in transfection studies where transfection of Bcl-2 into an interleukin-3 dependant cell line resulted in increased cell survival following interleukin-3 withdrawal (Vaux et al, 1988). It was later shown that the prolonged cell survival in these transfected cells was due to the ability of Bcl-2 to inhibit apoptosis (Hockenbery et al, 1990). Since these initial studies, a large number other proteins sharing sequence homology to Bcl-2 have been isolated. Collectively these are known as the Bcl-2 family proteins, and both pro- and anti-apoptotic members have been isolated. Promoters of apoptosis include Bad, Bax and Bak amongst others; inhibitors include Bcl-2 and Bcl-XL, as well as others; pro and anti-apoptotic members of the Bcl-2 family, and their roles in the apoptotic pathway are reviewed by Gross et al, 1999.

Bcl-2, along with some other Bcl-2 family members has the ability to (a) form dimers with other members of the Bcl-2 family, (b) bind to non-homologous proteins and (c) form ion channels/pores. The ability of Bcl-2 to form heterodimers with other members of the Bcl-2 family is thought to be a major mechanism through which apoptosis is regulated. In 1993 Oltvai et al discovered that Bcl-2 was capable of heterodimerizing with the pro-apoptotic
protein Bax, and it was suggested that the ratio of Bcl-2:Bax may influence the susceptibility of a cell to undergo apoptosis.

Both Bcl-2 and Bcl-XL have been reported to be capable of binding to various unrelated proteins, including amongst others, Apaf-1, the caspase-binding Death Effector Domain (DED)-containing proteins Bap31 and MRIT, the protein kinase Raf-1, the Ca\(^{2+}\) activated phosphatase calcineurin, the HSP70 molecular chaperone regulator BAG-1, and the p53 binding protein 53BP-2 (reviewed by Reed, 1998), it is thought (although not proven) that these anti-apoptotic proteins may therefore protect the cell from effects of Ca\(^{2+}\) overload, heatshock, and mitochondrial poisoning.

Finally, various members of the Bcl-2 family have been shown to be capable of forming ion channels, at least in vitro (reviewed by Tsujimoto and Shimizu 2000). The formation of these channels may result in cytochrome c release from the mitochondria, resulting in the activation of a protein known as Apaf-1, which in turn cleaves caspase-9, initiating the cascade of caspase cleavage that results in apoptosis.

As mentioned, cells are committed to undergo apoptosis once the execution caspases are activated; this is achieved by cleavage of the pro-form by the initiator caspases. As apoptosis is irreversible following this, it is important that that the activation of the execution caspases is tightly controlled. The Bcl-2 family are thought to be responsible for this control although the methods by which this is achieved is still uncertain.
The mitochondria is thought to play an essential role in apoptosis, and the fact that Bcl-2 is a membrane protein located mainly on the outer layer of the mitochondrial membrane, suggests a role of Bcl-2 in preventing the mitochondrial changes that lead to apoptosis. During apoptosis, there is an influx of cytochrome c from the mitochondria into the cytosol, once in the cytosol, cytochrome c binds to Apaf-1, this complex recruits and induces the self-cleavage of caspase-9, therefore triggering the activation of the caspase pathway. Overexpression of Bcl-2 has been shown to prevent the release of cytochrome c into the cytosol (Yang et al, 1997) therefore blocking caspase activation and the apoptosis process.

1.4.3.2 Bax. Bax is a pro-apoptotic member of the Bcl-2 family and, as mentioned, has been shown to be capable of forming heterodimers with Bcl-2. Unlike Bcl-2, Bax has been shown in some cell lines to reside largely in the cytosol, and is thought to translocate to the mitochondrial membrane in response to various apoptotic stimuli; forced dimerisation of Bax in the cytosol is also thought to result in translocation to the mitochondria (Gross et al, 1998). Bax has been shown to directly induce the release of cytochrome c from the mitochondria into the cytosol (Eskes et al 1998; Jürgensmeier et al, 1998) therefore activating the apoptotic pathway. Release of cytochrome c is thought to result from the insertion and tetrameric oligomerisation of Bax into the outer mitochondrial membrane, resulting in the formation of an ion-channel (Saito et al, 2000); this process is thought to be induced by Bid. Bid is cleaved and therefore activated by caspase-8, and following this activation the C-terminal fragment of Bid translocates to the nucleus, where it triggers Bax oligomerisation (Roucou et al, 2002).
1.4.3.3 Bcl-X\textsubscript{L}  Bcl-X\textsubscript{L}, like Bcl-2 is a anti-apoptotic protein and when co-expressed with Bax in cells can abrogate the pro-apoptotic effects of the Bax protein. Bcl-X\textsubscript{L} has been shown to inhibit the release of cytochrome c and suppress the proteolytic cleavage of caspase-3. However when exogenous cytochrome c was added, Bcl-X\textsubscript{L} failed to suppress the activation of caspase-3, suggesting that the action of Bcl-X\textsubscript{L} lies upstream of cytochrome c release (Jürgensmeier et al, 1998).

Like many Bcl-2 proteins, the structure of Bcl-X\textsubscript{L} suggests that the protein may be capable of forming pores or ion channels, it is suggested that the anti-apoptotic Bcl-2 family members generally form small closed channels, whilst the pro-apoptotic family members, such as Bax, form larger, open channels (reviewed by Schendel et al, 1998). Contradicting evidence for the role of Bcl-X\textsubscript{L} in preventing apoptosis however, comes from experiments showing that Bcl-X\textsubscript{L} can bind to and sequester Apaf-1 (Hu et al, 1998), therefore preventing Apaf-1 from binding to, and activating caspase-9.

Finally Bcl-X\textsubscript{L} has been reported to be associated with the mitochondrial PT pore. This pore is thought to play a vital role in the loss of mitochondrial membrane potential ($\Delta \psi$) and cytochrome c release that occurs during apoptosis. The PT pore is made up of inner and outer membrane proteins, such as adenine nucleotide translocator (ANT) and voltage-dependant anion channel (VDAC) respectively. Bcl-X\textsubscript{L} has been shown to close the VDAC channel on liposomes whereas Bax appears to open the channel (reviewed by Tsujimoto and Shimizu 2000). Bax has also been shown to interact with ANT, which when opened, induces depolarisation of the inner membrane.
1.4.4 Apoptosis in the Colon

1.4.4.1 Apoptosis in the Normal Colon. As previously discussed, colonic epithelium stem cells found in crypt bases, undergo differentiation and migration up the crypt wall, to the luminal surface where they are lost in a process taking only three to eight days. Cell turnover within the colonic epithelium is high, and apoptosis plays a vital role in the loss of cells at the luminal surface, the fact that apoptotic rates approximately balance cell proliferation rates, suggests that apoptosis is responsible for maintaining cell population homeostasis within the colon.

Within normal colonic mucosa, we would expect to see high levels of Bcl-2 in the base of the crypts, were undifferentiated stem cells are located, and high levels of Bax or Bak at the luminal surface, where cells are lost by apoptosis. Indeed, increased levels of Bak have been found on the luminal surface (Moss et al, 1996) whilst Bcl-2 expression has been shown to be localised in the base of the crypts (Flohil et al, 1996).

Detachment-induced cell death (DICD) is a recognised form of cell death within intestinal epithelial cells, however it is controversial as to whether or not the cells detach before dying. Following detachment, apoptosis is seen in mouse epithelial cells, and this is accompanied by activation of the caspase cascade and subsequent DNA fragmentation (Grossmann et al, 1998).

In addition to DICD, Studies within the human colonic cell line CaCo-2 have shown apoptosis to be linked to colonic cell differentiation, which coincided with down-regulation of Bcl-2 and Bcl-XL (Litvak et al, 1998). Finally apoptosis has been shown to be induced in the colon in response to lack of nutrients for cellular metabolism; butyrate acts as a fuel to colonic
epithelial cells, and the removal of butyrate leads to increased expression of Bax and rapid apoptosis within colonocytes (Hass et al, 1997).

Apoptosis is essential for the maintenance of normal colonic morphology and function and an increase or decrease in the normal apoptotic rate can lead to a variety of pathological conditions.

1.4.4.2 Apoptosis and Colonic Neoplasia. As colorectal cancer results from a deregulation of factors normally controlling cell growth and survival, the possible role of abnormal apoptosis in carcinogenesis is obvious. It would seem that neoplasia could result from an increase in cell proliferation, a decrease in apoptosis, or a combination of both factors. Therefore it could be supposed that apoptotic levels in colonic neoplasia would be decreased in comparison to normal tissue. However, this does not appear to be the case. Hawkins et al, 1997 demonstrated increased apoptosis in colorectal adenomas and carcinomas in comparison to normal tissue, furthermore, apoptosis increased with Dukes’ stage.

Numerous studies have been carried out on the Bcl-2 family expression in neoplasia, however reports vary, and in addition staining has shown great variability between tumours. Bcl-2 expression was reported to be upregulated in colonic adenomas in comparison to normal tissue, and to be expressed throughout the colonic crypts rather than being confined to the crypt base (Yang et al, 1999; Flohil et al, 1996). However Bcl-2 expression appears to be decreased in carcinomas in comparison to adenomas (Hawkins et al 1997), and carcinomas frequently show no Bcl-2 immunoreactivity (Flohil et al, 1996; Maurer et al, 1998), these
results may indicate that Bcl-2 may play a role in the early stages of neoplasia, but may be lost in colonic carcinomas.

In contrast to this, Bcl-X\textsubscript{L}, has been shown to be increased in colorectal cancers but not in adenomas or well-differentiated carcinomas (Maurer et al, 1998). This suggests that Bcl-X\textsubscript{L} may play a more important role in the late stages of carcinogenesis than Bcl-2. Furthermore Bcl-X\textsubscript{L} has been shown to exert a stronger anti-apoptotic effect on cells than Bcl-2 (Gottschalk et al, 1994).

Bax has been reported to be elevated in rats with induced colonic tumours (Hirose et al, 1997) and is reported to be mutated in over 50% of colorectal cancers with the microsatellite mutator phenotype (MMP+) in addition, the expression of Bax within colorectal carcinomas is diffuse, rather than localised to the luminal surface (Maurer et al, 1998). Bak, a pro-apoptotic member of the Bcl-2 family has been shown to be generally down regulated in colorectal carcinomas (Krajewska et al, 1996; Maurer et al, 1998). It is therefore possible that Bcl-X\textsubscript{L}, Bak and possibly Bax may play a more important role in carcinogenesis than Bcl-2, however the overall increase in apoptosis in colorectal carcinomas in comparison to normal tissue, suggests that cell proliferation may be a more important factor than the inhibition of cell death.

1.4.4.3 Apoptosis and IBD. During idiopathic IBD there is breakdown of the epithelial layer, it is possible that enhanced apoptosis may play a role in this. Studies in patients suffering from UC have shown increased apoptosis rates in the colonic mucosa of patients suffering from UC, in comparison to normal controls. These rates were higher still in patients that
ultimately required surgery rather than those that could be managed by medication alone (Hagiwara et al, 2002).

Polymorphonuclear neutrophils (PMN) are seen to accumulate in the tissue of patients with IBD. This accumulation may be due to a decrease in apoptosis of PMN, resulting in their prolonged survival and therefore excessive accumulation. Ina et al, 1999(a), found that mucosa taken from patients with IBD had increased levels of both granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage CSF (GM-CSF), and that when PMN were exposed to G-CSF and GM-CSF in culture, a dose dependant decrease in apoptosis occurred. These experiments suggested that decreased apoptosis might be important in the accumulation of PMN in IBD. CD is characterised by the accumulation of activated T-cells in the colonic mucosa, and T cells from CD show inhibition of normal apoptosis. This decrease in T cell apoptosis is accompanied by elevated Bcl-2 expression (Boirivant et al, 1999) and a decrease in Bax expression (Itoh et al, 2001). It has been demonstrated that T cells from CD are more resistant to Fas and nitric oxide induced apoptosis than normal or UC cells (Ina et al, 1999(b)). These results suggest that in CD, T cells may accumulate inappropriately, due to the imbalance between cell proliferation and apoptosis. This T cell accumulation may lead to inappropriate immune responses and chronic inflammation.

Alterations in the susceptibility of T cells to apoptosis in UC are less clear; Bu et al (2001) found a marked reduction in the levels of apoptotic T cells in the lamina propria of patients suffering UC in comparison to normal controls; in contrast to this study, Sträter et al (1997) observed an increase in apoptosis within the lamina propria in UC sufferers.
Within UC colonic mucosa however, decreased Bcl-2 and increased Bax levels are found. This in turn may result in increased apoptosis, therefore leading to the breakdown of the mucosa. This correlates with other studies, which demonstrate increased apoptosis within the colonic epithelium in active UC (Sträter et al., 1997; Arai et al., 1999; Iwamoto et al., 1996).

Finally, Fas is expressed in the epithelial cells of normal mucosa, as well as in mucosa from UC and CD (Ueyama et al., 1998). Strong expression of Fas-L by infiltrating CD3 lymphocytes is found in UC lesions (but not CD), suggesting that the Fas signalling pathways may further contribute to the induction of apoptosis and mucosal breakdown seen in UC.

As previously discussed, both UC and CD predispose to neoplasia. The deregulation of apoptosis is an important mechanism in the development of colorectal cancer (reviewed by Butler et al., 1999 and Ramachandran et al., 2000) and mutations, or altered expression in members of the Bcl-2 family are of considerable interest in unravelling the possible pathways by which these colonic tumours may develop. The accelerated cell turnover and associated mutations found in both UC and CD are likely to predispose to DNA damage and hence contribute to the increased risk of colorectal cancer.

1.5 Cyclooxygenase

1.5.1 Introduction. NSAIDs reduce inflammation by inhibiting the production of prostaglandins. Prostaglandins are synthesised from arachadonic acid, and the key enzyme involved in this process is cyclooxygenase (Cox). Cox is involved in the first two steps of prostaglandin synthesis, the first step involves the oxidation of arachadonic acid to form PGG2, and this is then subsequently reduced to give rise to PGH2. PGH2 is subsequently
transformed into the primary prostanoids through various other enzymatic and non-enzymatic mechanisms. Cyclooxygenase is the target enzyme for a group of drugs known as non-steroidal anti-inflammatory drugs (NSAIDs) (Vane 1971). As the name suggests these are anti-inflammatory agents that are currently used to treat various medical conditions. NSAIDs work by inhibiting the action of the Cox enzymes, this may be achieved by irreversible covalent modification of the enzyme (as is the case with aspirin), however most NSAIDs (including indomethacin) cause reversible or irreversible inhibition of Cox by competing with arachadonic acid for the Cox active site.

Following the initial purification of Cox in 1976 (Miyamoto et al), a second isoform was isolated in the early 1990's (Simmons et al, 1989; Kujubu et al, 1991) and the two isoforms are known as Cox-1 and Cox-2 respectively. Cox-2 was found to be an inducible enzyme, which is expressed in response to various inflammatory and mitotic stimuli, whilst Cox-1 is considered to be the constitutively expressed isoform, however this is an over-simplified view as Cox-2 has been shown to be constitutively expressed within the brain and spinal cord (reviewed by Vane et al, 1997) and there are several lines of evidence suggesting that Cox-1 contributes to the inflammatory process. This will be discussed below.

Structurally, Cox-1 and Cox-2 are very similar in structure and catalytic activity (reviewed by Vane et al 1997) however whilst both Cox-1 and -2 are equally capable of catalysing the oxidation and reduction of arachadonic acid, Cox-2 is able to utilise a wider range of fatty-acids as substrates than Cox-1. The three-dimensional x-ray crystal structures of Cox-1 and Cox-2 can be superimposed on each other and the structures of the binding and catalytic sites
are identical with the exception of two amino-acid residues. In both cases the same amino acid substitution is found, where Ile in Cox-1 is exchanged for Val in Cox-2.

These two differing amino-acid substrates alone are thought to play an important role in the differences of the two isoforms, and various experiments, where point mutations of Ile to Val in Cox-1 results in the biochemical phenotype of Cox-2 (and vice-versa) support this hypothesis (Wong et al, 1997; Gierse et al, 1996).

1.5.2 Cyclooxygenase-1. Cox-1 is constitutively expressed within most tissues and is responsible for producing prostglandins (eg prostacyclin) that maintain the epithelial barrier functions in the stomach and intestine. The gastric cytoprotective role of these prostaglandins is thought to be due to their vasodilation properties, which increase blood flow; and inhibition of Cox-1 has been shown to result in gastric ulceration and haemorrhage. Within the intestine, Cox-1 is thought to be responsible for producing prostaglandins that are important in promoting crypt stem cell survival and proliferation (Cohn et al, 1997). The production of prostaglandins by Cox-1 is also important in maintaining blood flow in the compromised kidney (McGiff and Wong 1979) and therefore treatment of patients suffering kidney abnormalities with NSAIDs can result in renal ischemia (McCarthy et al, 1982).

Cox-2 was originally assumed to be solely responsible for inflammatory events, however various lines of evidence suggest a role for Cox-1 in inflammation. Cox-1 expression is seen at inflammation sites in patients suffering from rheumatoid arthritis, and osteoarthritis (Siegle et al, 1998) and Cox-1 has been shown to produce prostaglandins in response to Helicobacter pylori gastritis (Jackson et al, 2000). Furthermore, in various experiments, reduction of pain
in rat models on inflammation was only obtained with selective Cox-2 inhibitors when they were used at a great enough concentration to also cause inhibition of Cox-1 (Wallace et al, 1998), and finally, the Cox-1 specific inhibitor, SC-560, has been shown to be equally effective as Cox-2 specific inhibitors, in limiting prostaglandin synthesis in the rat paw (Smith et al, 1998).

In contrast to the inhibition of Cox-1 function in adult models, the production of Cox-1 knockout mice in 1995 by Langenbach et al showed no obvious gastrointestinal abnormalities, despite the fact that Cox-1 levels were only 1% of that observed in wild-type mice. Compensation by Cox-2 was eliminated, as levels were equivalent to those found in the wild-type mice. It was concluded that other cytoprotective mechanisms took over in the absence of prostaglandin production. As expected, no significant renal pathology was seen, confirming that Cox-1 expression is not necessary for maintaining normal renal blood flow. Cox-1 null mice however, did show decreased platelet aggregation in response to arachadonic acid treatment. Cox-1 is responsible for generating the precursors to thromboxane, which in turn leads to platelet aggregation (Schafer 1995) and this is the rationale behind the prevention of thromboembolic disease with "half an aspirin a day". Finally, expression of Cox-1 was shown to be important in foetus survival as 90% perinatal mortality was seen in pups born to homozygous Cox-1 null parents.

1.5.3 Cyclooxygenase-2. Whilst Cox-2 is considered to be the inducible isoform, expressed in response to mitotic and inflammatory stimuli, it is constitutively expressed in some normal (i.e. non-inflammed) tissues. Most noticeably is within the macula densa of the normal kidney, (reviewed by Breyer and Harris 2001) where it is thought to play an important role in
increasing the production of renal rennin in response to dietary sodium restriction (Harris et al, 1994; Harding et al, 1997). Cox-2 expression is increased during ovulation, most likely in response to luteinizing hormone and gonadotropin-releasing hormone, and inhibition of Cox-2 prevents ovulation (Wong et al, 1992). Cox-2 expression is also associated with embryo implantation (Chakraborty et al, 1996) and prostaglandins produced from Cox-2 may be involved in the initiation of labour, as stimulation of Cox-2 caused premature labour in sheep (McLaren et al, 1996). It is possible that preterm labour could occur as a result of intrauterine infection leading to induction of Cox-2 expression (Spaziani et al, 1996). Cox-2 is constitutively expressed within the brain where it is up-regulated by both normal and abnormal (convulsive) nerve activity (Yamagata et al, 1993).

Expression of Cox-2 is greatly increased at sites of inflammation (Vane et al, 1994), where it is responsible for the production of pro-inflammatory prostaglandins responsible for the vasodilation, fever and pain that are associated with infection. In addition to exacerbating inflammation, Cox-2 may also play a role in limiting the inflammatory response (Wallace et al, 1998). In an experimental rat model, following initiation of inflammation, Cox-2 was found to produce "E-type" prostaglandins, however 48 hours later 15-deoxy-\(^{\Delta 12,14}\)PGJ\(_2\) production was seen instead of PGE\(_2\); treatment with the Cox-2 specific inhibitor NS-398 within an hour of injury reduced inflammation, but treatment with NS-398 24 hours after injury further exacerbated inflammation. Furthermore, animal models have suggested that inhibition of Cox-2 may slow the healing process of gastro-intestinal ulcers (Schmassmann et al, 1998).
As expected, Cox-2 null mice suffered severe nephropathology and reproductive defects (Dinchuk et al, 1995). Female Cox-2 mice were generally infertile, due to a lack of ovulation. Implantation and decidualization were also defective (Lim et al, 1997).

1.6 Non-Steroidal Anti-Inflammatory Drugs

Non-steroidal anti-inflammatory drugs are a group of drugs commonly used for their analgesic, antipyretic and anti-inflammatory properties. The predominant mechanism by which they act is presumed to be through the inhibition of the cyclooxygenase enzyme, a key enzyme involved in the production of prostaglandins from arachidonic acid. NSAIDs work by inhibiting Cox (either through competitive binding or covalent modification) and this in turn results in an inhibition of prostaglandin production, therefore reducing the inflammatory response.

The first indication of the chemopreventive properties of NSAIDs came in 1983 when Waddell and Loughry observed a virtual disappearance of rectal polyps in a group of patients suffering from Gardner’s syndrome in response to sulindac treatment. This, together with the observations of Pollard and Luckert (1983) that indomethacin reduced the number of carcinogen-induced tumours in rats, provided the earliest evidence for the chemopreventive potential of the NSAIDs. Following this, Kune et al (1988) demonstrated a decreased risk of colorectal cancer amongst adults who regularly used aspirin. Later studies carried out in the 1990’s suggested that regular use of aspirin results in a 40-50% lower risk of colorectal cancer (Thun et al, 1993). Importantly, NSAIDs have also been shown to exert similar antitumorigenic properties in human colorectal adenomas indicating that the mechanism of action may be early on in the process of carcinogenesis (Logan et al, 1993). Various epidemiological
studies into the chemopreventative potential of various NSAIDs have been carried out since these initial studies, and the vast majority support the hypothesis that NSAIDs reduce the occurrence of colorectal tumours (reviewed by Muir and Logan, 1999).

The ability of NSAIDs to induce apoptosis in colorectal epithelial cells was first demonstrated by Pasricha et al in 1995, who found that the number of apoptotic cells in the epithelium from patients with FAP increased significantly following treatment with sulindac. This was the first indication that the chemopreventive mechanism of these drugs may be due to their ability to increase apoptosis, therefore helping to minimise the accumulation of abnormal cells that arise in a tumour. Other groups, using various models have since demonstrated the induction of apoptosis in colonic epithelial cells by NSAIDs in human colonic cancer cell lines (Shiff et al, 1995; Hong et al 1998), animal models (Rao et al, 1995; Mahmoud et al, 1998) and patients with FAP (Keller et al, 1999).

Whilst it is generally agreed that NSAIDs induce apoptosis in malignant colorectal epithelial cells, the effects on cell proliferation are less clear. Whilst Hanif et al (1996) and Shiff et al (1995) reported that sulindac sulfide inhibited proliferation of colonic cancer cell lines, proliferation value reported was assessed by the number of cells at a certain time point, compared to the cell number plated, therefore the reduction in cell number may in fact be a result of increased apoptosis rather than a decrease in cell proliferation. Sawaoka et al (1998), measured cell growth by BrdU incorporation, and found that the Cox-2 inhibitor NS-398 did suppress cell replication in gastric cancer xenografts in nude mice. In contrast to this however, Pasricha et al (1995) reported no decrease in colonic cell proliferation in FAP
patients treated with sulindac. Further studies are needed in order to clarify the effects of NSAIDs on colonic cell proliferation.

A further mechanism by which NSAIDs may inhibit tumour growth is by inducing cell cycle quiescence. Indeed, NSAIDs have been found to induce G0/G1 cell cycle arrest (Shiff et al, 1996; Piazza et al, 1997; Hanif et al, 1996) (sulindac sulfide induced G2/M cell cycle arrest in the HCT-15 cell line (Hanif et al, 1996)).

As both Cox-2 and prostaglandins have been shown to be elevated in colonic adenomas and carcinomas (Maekawa et al, 1998) and prostaglandins have been shown to stimulate proliferation, reduce apoptosis and increase levels of bcl-2 within colonocytes, it was originally presumed that NSAIDs where chemopreventive due to their ability to inhibit Cox and therefore reduce levels of prostaglandins, however there are several lines of evidence that challenge this. Firstly, NSAIDs are capable of inducing apoptosis in colonic cancer cell lines that produce no detectable Cox transcripts or prostaglandins (Hanif et al, 1996), secondly, the sulfone metabolite of sulindac which does not inhibit Cox-1 or Cox-2 does prevent carcinogen induced tumour formation in a rat model (Piazza et al, 1997) and thirdly, the ability of a NSAID to prevent tumour formation, does not necessarily correlate with it's ability to inhibit prostaglandin synthesis (Piazza et al, 1995).

Various alternative mechanisms have been put forward to explain the Cox-independent chemopreventive effects of NSAIDs (figure 1.6). Firstly, aspirin and sulindac have been shown to inhibit NF-kB activation (Kopp and Ghosh, 1994) and there is some evidence to suggest that this is achieved by direct binding too, and inhibition of IkB kinase β. Normally
this enzyme will phosphorylate Ik-B therefore targeting it for degradation and consequently activating the NF-kB pathway. Following activation, NF-kB translocates to the nucleus where it activates the transcription of various genes. However, indomethacin, which does not inhibit IkB kinase (Yin et al, 1998), is equally capable of preventing colonic neoplasia and causing regression of adenomas in FAP patients, suggesting that this alone is unlikely to be a key mechanism for NSAID chemoprevention.

A further possible target of the NSAIDs are a group of nuclear hormone receptors known as the peroxisome proliferator activated receptors (PPAR), of which there are three isoforms known as α, γ, and δ. Interestingly, various NSAIDs have been shown to be capable of directly binding too and therefore activating PPAR’s (Lehmann et al, 1997; He et al, 1999). Activation of PPARγ has been shown to reduce tumour cell growth and induce cellular differentiation (Sarraf et al 1998; Kitamura et al, 1999). PPARδ is thought to be a down-stream target of the β-catenin pathway, which becomes active as a result of APC mutations in colonic cancer. Sulindac has been shown to directly bind to and inhibit the DNA binding ability of PPARδ (He et al, 1999). Whilst the target genes of PPARδ are as yet unknown, PPARδ is known to be over-expressed in colorectal cell lines (Gupta et al, 2000) and therefore over-expression of this particular PPAR isoform may contribute to a malignant phenotype. In conclusion, whilst there is evidence for interaction of NSAIDs with PPARs, the roles of the three PPAR isoforms in the development of colonic cancer are as yet unknown and the importance of this potential chemopreventive pathway is therefore unclear.

A possible pathway for the pro-apoptotic effects of NSAIDs is through the activation of the pro-apoptotic gene Bax. Inhibition of Bcl-XL by NSAIDs leads to an increase in the Bax/Bcl-XL ratio within the cell, which in turn favours cellular apoptosis.
Finally, both sulindac and the Cox-2 specific inhibitor, etodolac, have been shown to increase the level of APC mRNA expression in rats treated with the carcinogen azoxymethane (AZO) (Kishimoto et al, 2000). In normal cells APC binds to β-catenin. In the absence of APC, β-catenin forms a transcription complex with TCF-4, and this complex can then activate the c-myc oncogene and induce PPARδ expression.

As described, there are various mechanisms proposed to explain the chemopreventive effects of NSAIDs, these include inhibition of prostaglandin synthesis, inhibition of Cox-2, activation of NF-κB, interaction with PPARs, alterations in the pro:anti apoptotic gene ratio, and modification of gene expression.

In reality, it is probably combination of various mechanisms that together play a role in the action of NSAIDs on colonic epithelial cells and further work is needed to fully understand the mechanisms by which NSAIDs exert their effects.

Whilst it is now generally accepted that NSAIDS are chemopreventive, their use for this purpose is hampered as a result of the known side-effects of prolonged NSAID administration, in particular with regard to their gastric and renal toxicity. The identification of the Cox-2 isoform has paved the way for the development of Cox-2 specific inhibitors. As Cox-2 is the isoform commonly over-expressed in colonic tumours, it was proposed that these new drugs could be used as chemopreventive agents without the toxic side-effects of the non-specific Cox inhibitors.
NSAIDs have been shown to (A) inhibit the NF-kB pathway by inhibiton of IkB kinase, this enzyme normally phosphorylates IkB, targeting it for degradation and consequently activating the NF-kB pathway; (B) Inhibit COX, therefore reducing the production of prostaglandins; (C) Inhibit Bcl-XL, therefore increasing the ratio of Bax/Bcl-XL, which in turn favours apoptosis; (D) Increase APC expression, therefore preventing the translocation of β-catenin to the nucleus; (E) Activate PPARα/γ, and (F) prevent the PPARδ/RXR complex binding to DNA. (adapted from Gupta and DuBios, 2001)
Figure 6. Mechanisms of chemoprevention by NSAIDs.
1.7 Peroxisome Proliferator Activated Receptors

1.7.1 Introduction. Peroxisome proliferator-activated receptors (PPARs) were first described by Isseman and Green in 1990 as a result of a search for the molecular target of hepatic peroxisome proliferating agents in rodents. To date three subtypes of PPARs have been characterised, and are known as PPARα, PPARδ and PPARγ. All three subtypes belong to the nuclear receptor superfamily and are ligand activated transcription factors.

PPARs regulate gene expression by binding to PPRE response elements (PPRE) in the promotor region of target genes. PPARs bind to PPRE as a heterodimer with the receptor for 9-cis retinoic acid, RXR (retinoid X receptor). Binding of a ligand to this heterodimer then leads to a conformational change resulting in stabilization of the complex and the formation of a binding cleft; this leads to the recruitment of co-activators, and the initiation of transcription.

The three PPAR isoforms share a certain degree of substrate specificity. All three forms have been shown to be activated by various fatty acids and fatty acid derivatives, such as prostaglandins and eicosanoids, suggesting an important role in lipid metabolism. In addition, PPARs have been shown to be the targets of various synthetic compounds that are used in the treatment of diabetes and dyslipidemia.

In addition to the common substrates, each PPAR isoform is activated by various differing ligands, these, together with a more detailed description of their individual physiological functions are discussed below.
1.7.2 PPARα. PPARα was the first member of the PPAR family to be cloned (Isseman and Green, 1990) and, like PPARδ and γ, is involved in lipid metabolism. PPARα has been shown to have a critical role in the cellular uptake, activation and β-oxidation of fatty acids (Kersten et al, 1999; Lee et al, 1995).

The target genes of PPARα are generally involved in the catabolism of lipids, including fatty acid uptake into the cells, binding of fatty acids within the cell and oxidation of fatty acids within various organelles such as mitochondria, peroxisomes and microsomes (Kersten et al, 2000). In addition PPARα is involved in the synthesis of lipoproteins. The actions of PPARα have mainly been studied in hepatocytes as PPARα levels are highest in rodent liver, however significant expression is also seen in rodent kidney, skeletal muscle, and heart. Low-level expression has been detected in the brain, lung and spleen (Braissant et al, 1996). Similar expression patterns are seen in human tissue, although rat and human PPARα displayed differential responsiveness to various ligands (Mukherjee et al, 1994, Lawrence et al, 2001)

A diverse range of chemicals can activate PPARα, including fatty acids, such as linoleic acid and arachandonic acid, fibrates, eicosanoids and endogenous ligands. Following the discovery that PPARα was a nuclear receptor for the leukotriene B4 (LTB4) it was suggested that this isoform may play a role in inflammation. PPARα is thought to control the inflammatory response through the degradation of fatty acids and their derivatives, ensuring that LTB4 is cleared from the liver. Experiments showing prolonged immune responses in PPARα deficient mice further supported a role in inflammation.
PPARα may also play a part in the inflammatory process by interfering with the NF-kB signalling pathway. NF-kB activation was shown to correlate with the expression of genes involved in the inflammatory response such as interleukin-6 (IL-6), interleukin-2 (IL-2), TNFα and Cox-2 (Spencer et al, 1997). Treatment of macrophages with PPARα ligands was shown to reduce NF-kB activity (Poynter et al, 1998), therefore down-regulating the expression of pro-inflammatory cytokines (Staels et al, 1998). PPARα has been shown to reduce NF-kB activity both through the induction of IkBa expression (Delerive et al, 2002) and through direct binding with the p65 subunit of NF-kB.

Due to its ability to interfere with the NF-kB signaling pathway, PPARα may play a role in the induction of apoptosis. Ligand activation of PPARα within macrophages led to an increase in TNFα induced apoptosis (Chinetti et al, 1998). NF-kB has been shown to be capable of protecting cells from TNFα-induced cell death, and inhibition of NF-kB by PPARα may explain why simultaneous treatment of the macrophages with TNF-α and a PPARα ligand led to an increase in apoptosis.

Much of the work investigating the functions of PPARα has been carried out in rodents, and during these experiments it was discovered that animals chronically fed peroxisome proliferators (PP) developed hepatocellular carcinomas (Reddy et al, 1979), further experiments using the PPARα-null mouse showed that this was a PPARα-mediated process (Peters et al, 1997). Whilst the exact mechanism by which PPARα leads to carcinogenesis is as yet unclear, various theories have been put forward. Firstly treatment of mice with the PPARα ligand Wy-12 643 led to an increase in the mRNA and protein levels of CDK-1, CDK-4, cyclin D1 and c-myc (Peters et al, 1998), all of which have been shown to be present at
elevated levels in mice hepatocellular carcinomas (Yerly-Motta et al, 1999). Hepatocyte growth factor (HGF) levels are decreased in response to PPARα activation, which may upset the normal balance of cell proliferation, resulting in the promotion of tumorigenesis (Motoki et al, 1997).

In contrast to the suggested induction of apoptosis through inhibition of the NF-kB pathway, experiments in rat hepatocytes have suggested that PP are capable of suppressing cell death in response to apoptotic stimuli. (Roberts et al, 1998). Furthermore, Christensen et al (1998) showed that Nafenopin inhibited apoptosis in mouse hepatocytes through a PPARα dependant pathway, which resulted in alterations to the normal regulation of Bcl-2 and Bak.

Together, this data suggests that activation of the PPARα pathway leads to the development of hepatocellular carcinoma in rodents as a result of altered cell cycle and cell survival gene expression, leading to increased cell proliferation and the suppression of apoptosis. However these effects are not observed in human liver, this could be due to the fact that PPARα mRNA is known to be expressed at approximately a 10 fold higher level in rodent, than in human liver (Tugwood et al, 1998) (Palmer et al, 1998). Additionally, a mutated form of PPARα, known as hPPARα8/14) has been found to be present in all human liver samples examined to date (Tugwood et al, 1996). This alternatively spliced variant results in the deletion of exon 6, which brings a stop code into the reading frame and results in a truncated protein containing 174 amino acids rather than the normal 409. A second variant of PPARα (hPPARα-6/29) also found in human liver, is a full-length receptor with four amino acid changes from the wild-type PPARα sequence. This particular isoform binds to DNA but is not activated by PP, and acts as a dominant negative repressor of wild-type PPARα (Roberts et al, 1998). Furthermore,
Woodyat et al (1999) found that each of 22 unrelated human individuals had an inactive peroxisome proliferator response element (PPRE) in the human acyl CoA oxidase gene. These combined factors may in part explain why PP do not have the same hepatocellular carcinogenic effects in humans as are seen in rodents.

1.7.3 PPARγ. Whilst initial studies suggested that, as expected, expression of PPARγ was highest in adipose tissue, recent studies have identified the colon as a further site of high expression (Auboeuf et al, 1997), with levels at their highest in the post-mitotic, differentiated epithelial cells (Mansen et al, 1996; Lefebvre et al, 1999). Activation of PPARγ has been shown to reduce tumour growth rate in mice, inhibit colonic cell growth through G1 cell cycle arrest, and induce differentiation in colonic cancer cell lines (Sarraf et al, 1998; Kitamura et al, 1999). Yang and Frucht (2001) showed that treatment of the human colonic carcinoma cell line HT-29 with a selective PPARγ ligand induced colonic cell apoptosis and decreased Cox-2 expression. Cox-2 is known to be a promoter of colonic cancer and so the ability of PPARγ to reduce Cox-2 expression provides a further possible mechanism to explain the anti-tumorigenic role of PPARγ.

However, two other studies have demonstrated a pro-tumorigenic role for PPARγ. APCmin/+ mice are a useful model which represent both human FAP and sporadic colonic cancer. When these mice were treated with two different thiazolidinediones (TZDs), (which are drugs used in the treatment of diabetes, and known ligands of PPARγ) a small increase in the number of colonic polyps was observed (Saez et al, 1998, Lefebvre et al, 1998). These discrepancies may be due to a number of factors, including species differences between mice and human PPARγ, the differentiation status of the colonic cells (activation of PPARγ was
been shown to stimulate polyp formation (well differentiated cells) but inhibit implanted
tumours (poorly differentiated cells)), or concentration of the PPARγ ligand.

In addition to genes involved in fatty acid transport and storage, further target genes of PPARγ
have been identified that are associated with cell proliferation and differentiation (Gupta et al,
2001), these include regeneration gene IA (RegIA), Neutrophil gelatinase-associated lipocalin
(NGAL) (involved in the negative control of the inflammatory response (Kjeldsen et al,
2000)), Gob-4 (associated with mature goblet cells (Komiya et al, 1999)) and keratin 20
(expressed in most differentiated intestinal mucosal epithelium (Calnek and Quaroni, 1993)).
Of these RegIA is of particular interest.

RegIA was originally identified as a secreted protein that could stimulate the production of
pancreatic β-cells and ductal cells (Kobayashi et al, 2000). Over expression of RegIA in mice
led to the development of multiple tumours (Yamaoka et al, 2000), additionally, RegIA has
been found to be over-expressed in a large percentage of colorectal tumours, where it
negatively correlates with differentiation status and patient survival (Rechreche et al, 1999;
Shinozaki et al, 2001; Macadam et al, 2000). Activation of PPARγ was found to repress
RegIA gene expression, which suggests that activation of the PPARγ pathway may reduce the
malignant potential of colonic cancer cells.

The non-steroidal anti-inflammatory drug indomethacin is known to act as an adipogenic
agent (Verrando et al, 1981; Knight et al, 1987). Whilst it was originally presumed that this
was due to its ability to inhibit Cox activity, the fact that the concentration of indomethacin
needed to induce adipocyte differentiation was 2-3 times greater than that needed to inhibit
Cox activity suggested that a different mechanism might be underlying. Following the
discovery that PPARγ was both necessary and sufficient to induce adipocyte differentiation;
and with the knowledge that prostaglandins (which are products of the Cox pathway) are
known ligands of PPARγ, the question arose of how could a Cox inhibitor (which reduces the
production of prostaglandins, therefore limiting the production of PPARγ ligands), induce
adipocyte differentiation? Lehmann et al (1997) showed that indomethacin and various other
NSAIDs were capable of directly binding to, and activating PPARγ. Furthermore, the same
group of NSAIDs could also act as ligands for PPARα.

The role that PPARγ plays in inflammation is debateable; whilst PPARγ ligands have been
shown to inhibit the expression of TNF-α, IL-6 and IL-1B in monocytes (Jiang et al, 1998)
and various other cytokines, 15d-PGJ2, which is not a particularly selective PPARγ ligand,
was shown to have the most pronounced effects on the inflammatory response, and anti-
inflammatory effects were only observed with some PPARγ ligands when they were used at
far higher concentrations than needed to bind and activate PPARγ (reviewed by Delerive et al,
2001). However, in contrast to these results, Chinetti et al (1998), demonstrated that
activation of PPARγ resulted in the apoptosis of macrophages, and that PPARγ, like PPARα,
inhibited NF-kB activity through the direct binding with the p65 subunit. Finally, treatment of
a mouse model of colitis with TZDs has been shown to result in a marked decrease in colonic
inflammation (Su et al, 1999). However, further studies are needed to clarify the role of
PPARγ on inflammation in vivo.
1.7.4 **PPARδ.** PPARδ, like PPARα and γ can be activated by both saturated and unsaturated fatty acids. Arachadonic acid has been shown to be capable of binding to, and activating PPARδ, even at low micromolar concentrations (Foreman et al, 1997). Various eicosanoids, including both PGA1 and PGD2 are known to act as ligands for PPARδ (Yu et al, 1995). PPARδ has been shown to be expressed in a wide variety of tissues with high levels in brain, adipose tissue and skin (Braissant et al, 1996). As yet, the specific target genes of PPARδ are unknown.

PPARδ is thought to be involved in both embryo implantation (Lim et al, 199) and development and neuronal signalling within the central nervous system (Braissant et al, 1998; Matsumoto et al 2001; Peters et al, 2000), however, since the discovery that induction of APC expression in HT-29 cells resulted in a reduction in the levels of PPARδ (He et al, 1999), and that PPARδ mRNA is frequently over expressed in colonic cancer cells (He et al, 1999; Gupta et al, 2000), there has been considerable interest in the role of PPARδ in colon carcinogenesis.

In normal tissue APC binds to a cytoplasmic protein known as β-catenin, which has important functions in cell adhesion and development. If APC is mutated then levels of cytoplasmic β-catenin become elevated, under these conditions β-catenin translocates to the nucleus, where it binds with T-cell factor 4 (TCF-4) forming a transcription complex. Targets of the β-catenin/TCF-4 transcription complex are genes involved in promoting cell growth and differentiation including c-MYC and cyclin D1 (He et al, 1998 Tetsu et al, 1999). PPARδ is thought to be down regulated by APC, as it is over-expressed in colonic cancer cells containing APC mutations, and indeed, the PPARδ promoter site has been shown to directly interact and bind to the β-catenin/TCF-4 complex (He et al, 1999).
Experiments carried out in nude mice showed that a PPARδ-/- cell line has a greatly decreased ability to form tumours, compared to PPARδ+/− and wild type cell lines (Park et al, 2001). These experiments suggested that PPARδ expression may contribute to a tumorigenic phenotype.

As mentioned, NSAIDs have been shown to be effective in reducing the risk of colonic cancer; as NSAIDs inhibit the action of the Cox enzymes, which are crucial for the production of prostaglandins, which in turn act as ligands for PPARδ, is the down regulation of PPARδ an alternative mechanism for the chemopreventive properties of NSAIDs? Furthermore, NSAIDs have been shown to prevent the activation of the PPARδ target genes by preventing the binding of the RXR/PPARδ transcription complex to DNA through an unknown mechanism (reviewed by Wu, 2000). The inhibition of PPARδ in colonic cancer cells enhanced the ability of NSAIDs to induce apoptosis. However further work investigating the target genes of PPARδ, and the mechanism of inhibition by NSAIDs is still needed.

1.8 5-Aminosalicylic Acid

Sulphasalazine was a drug initially used in the 1930’s for the treatment of rheumatic polyarthritis, however its use for the treatment of colitis was soon noted (Svartz et al, 1942). Further investigations showed sulphasalazine to be effective in achieving and maintaining remittance in cases of active colitis (Dick et al, 1964), however despite the obvious benefits of sulphasalazine, a major limitation of its use was the occurrence of side effects (Taffet et al, 1983).
Sulphasalazine was found to be a pro-drug complexing 5-aminosalicylic acid (5-ASA) with sulfapyridine. The 5-ASA component was found to be the therapeutic moiety (Azad Khan et al, 1977), which is released in the colon following cleavage of the diazo bond by intestinal bacteria, whilst the sulfapyridine (which was responsible for the majority of adverse side effects) simply functioned as a carrier. This gave rise to a generation of new 5-ASA containing compounds designed to deliver the therapeutically active 5-ASA moiety to the colon, without the toxic effects of sulfapyridine. This is achieved by coating the 5-ASA compound (otherwise known as mesalazine) to prevent early release in the small intestine, or by conjugating 5-ASA to a less toxic carrier which is cleaved at the site of action (e.g. balsalazine). Mesalazine (the coated form of 5-ASA) is the most commonly used compound in the treatment of ulcerative colitis.

The mechanism of action of 5-ASA is as yet unclear. Various possible mechanisms, including inhibition of lipoxygenase, inhibition of neutrophil degranulation, inactivation of nitric oxide, inhibition of interleukin-1 production and inhibition of NF-kB, have been suggested (reviewed Travis et al 1994). In addition, 5-ASA has been shown to have a wide range of anti-oxidant effects, which may help to protect the colonic epithelial cells from neutrophil induced oxidative damage (Simmonds et al, 1999).

5-ASA is structurally similar to 5-acetylsalicylic acid (better known as aspirin) and indeed there have been a number of reported cases of nephrotoxicity associated with 5-ASA use (Novis et al, 1988). The structural similarity of 5-ASA to aspirin raises the possibility of inhibition of Cox as a possible mechanism of action, and 5-ASA has been shown to interfere
with both the cyclooxygenase and lipoxygenase pathways (reviewed by Ireland and Jewell 1990).

Furthermore, if 5-ASA is structurally similar too, and may work through similar mechanisms as aspirin, could it also demonstrate the chemopreventive properties associated with the NSAID’s? Experiments carried out in patients suffering from colorectal cancer have shown that mesalazine enemas given for 14 days greatly increased the number of apoptotic cells within the malignant tissue (no effect was seen in normal colonic mucosa) however no effect was seen on cell proliferation (as measured by Ki-67 staining) (Bus et al, 1999). In contrast to this study, MacGregor et al (2000) found that both balsalazide and 5-ASA inhibited proliferation in colonic cancer cell lines. Whilst this was consistent with the induction of apoptosis in 5-ASA treated cells, no increase in apoptosis was seen in response to balsalazide treatment. This indicated that balsalazide was inhibiting colonic cell growth through a different mechanism.

Balsalazide has been shown to reduce the number of aberrant crypt foci (ACF) in rats treated with the carcinogen azoxymethane, by 60%; and a dose dependant reduction in tumour formation of up to 80% has been observed in an APC mouse model in response to balsalazide treatment (MacGregor et al, 2000). 5-ASA has also shown to effectively reduce tumour size and number in N-MethylNitrosourea treated rats (Narisawa et al, 2002). However, whilst there is evidence for the anti-proliferative effects of the 5-ASA containing drugs, colonic cancer cells lines are significantly less sensitive to these compounds than to the non-steroidal anti-inflammatory drug indomethacin.
Flow cytometric analysis of 5-ASA and balsalazide treatment in the colonic cancer cell line LS174T showed cell accumulation in the G0/G1 phase of the cell cycle, and a concomitant decrease in the number of cells in S phase, in response to treatment with 10mM 5-ASA. The same result is seen when cells are treated with indomethacin (again the effect was considerably less with 5-ASA, than with indomethacin treatment) (MacGregor et al, 2000) Treatment with balsalazide did not alter the cell cycle distribution, however it should be remembered that balsalazide consists of 5-ASA conjugated to a 4-aminobenzoyl β-alanine carrier, which, in vivo, is split to release the active 5-ASA moiety in the colon, and metabolism of the drug in a colonic cell line may not accurately reflect the in vivo situation.

Evidence to date, suggests that 5-ASA may indeed be chemopreventive in the case of colorectal cancer, albeit to a lesser extent than the conventional NSAIDs. Unlike NSAIDs, 5-ASA compounds have been shown to be relatively safe to use and free of severe side-effects even with long-term use, and could therefore potentially be used as chemopreventive agents, however studies have mainly been carried out in animal models, with very limited reports on the effects of 5-ASA on human colonic tissue in vivo, and in human colonic cell lines. Furthermore, the mechanisms underlying any possible chemopreventive properties are as yet unclear. Further work, investigating both the effects of and the underlying mechanism of actions of the 5-ASA compounds is needed, and this has formed a large part of my thesis.

1.9 Aims of Studies Presented in this Thesis

The data presented in chapters 3 to 5 of this thesis were designed to analyse the role of apoptosis and proliferation in the development and prevention of colonic neoplasia. Studies presented in chapter 3 were designed to analyse cell turnover in normal, inflamed and
neoplastic colonic tissue in order to gain insight into the roles that both proliferation and apoptosis may play during development of various pathological disorders known to effect the colon. The changes in cell turnover were correlated with alterations in the expression of two members of the Bcl-2 family of apoptosis regulators, these being Bax and Bcl-2; expression of these two genes is thought to play an important role in determining cell survival. The effects of 5-ASA treatment on cell turnover in cases of UC were also examined.

NSAIDs are known to be chemopreventive within the colon, however the mechanisms underlying this chemoprevention are unknown. The studies presented in chapter 4 were designed to examine the effect of NSAID treatment on various colonic adenocarcinoma cell lines, in particular with regards to effects on cell viability, the induction of apoptosis, and the alterations in the cell-cycle. Furthermore the effects of 5-ASA, which has recently been reported to also possess chemopreventive properties, was examined and compared to the conventional NSAID indomethacin. Susceptibility of colonic cell lines to induction of apoptosis and disruption of cell-cycle were compared to the differing tumour grade, differentiation state and cyclooxygenase expression between cell lines, in order to determine if the expression of Cox is necessary for induction of apoptosis by indomethacin, and whether differing stages of tumour development may be more sensitive to NSAID treatment.

Finally, studies were designed to examine possible pathways by which NSAIDs and 5-ASA may inhibit tumour growth. The Bcl-2 family of proteins are important regulators of apoptosis, and expression of the pro-apoptotic gene Bax, and the anti-apoptotic gene Bcl-XL following indomethacin and 5-ASA treatment were analysed to see if alterations in the ratio of these genes may induce susceptibility to apoptosis. The PPAR group of nuclear hormone
receptors have recently become an area of interest in NSAID research. It has been shown that NSAIDs are capable of binding to, and activating these receptors and this may induce transcription of various genes associated with cell growth, differentiation, or malignant phenotype. Studies analysing expression levels of the PPAR isoforms, Bax and Bcl-X<sub>L</sub> following indomethacin or 5-ASA treatment are presented in chapter 5.

Alterations in cell turnover and the predisposition to colorectal cancer which is seen in UC sufferers; along with the mechanisms through which NSAIDs, and in particular, 5-ASA may act to prevent this are discussed in chapter 6, along with suggestions for further study.

Therefore, to summarise, the aims of this thesis were:

1. To analyse cell turnover in normal, neoplastic and inflamed colonic mucosa, and to examine how changes in cell turnover correlate with Bcl-2 and Bax expression.
2. To analyse and compare cell turnover in quiescent and severe UC in order to gain further insight into the mechanisms that underlie the chronic inflammatory state, and breakdown of colonic mucosa, which are associated with this disorder.
3. To examine the effects of 5-ASA treatment on cell turnover in active UC, in order to shed light on the mechanisms by which this compound may help to induce remission of active disease.
4. To compare the chemopreventive potential of 5-ASA with the NSAID indomethacin in malignant human colonic cells, in particular with regards to effects on cell viability, apoptosis and cell-cycle distribution.
5. To explore the mechanisms by which chemoprevention by NSAIDs and 5-ASA may occur; in particular with regards to alterations to Bax, Bcl-X<sub>L</sub> and PPAR expression.
2.1 Materials

2.1.2. Reagents

HT-29 cells were obtained from the German Collection of Micoorganisms and Cell Cultures, Braunschweig, Germany; HCT-15 and SW480 cells were obtained from European Collection of Cell Cultures, Salsbury, UK. All cells were received as frozen aliquots. CaCo-2 cells were a kind gift from Andrew Collick, Medical Research Council, Leicester.

Dulbecco’s modified eagle medium (DMEM); RPMI 1640; Glutamine; Penicillin/streptomycin (p/s); Foetal calf serum (FCS), Trypsin/EDTA 1X, phosphate buffered solution (PBS) and Hank’s buffered salt solution (HBSS) were obtained from GibcoBRL, Paisley, UK. L-15 medium was purchased from BioWhittaker, Abingdon UK.

The anti-caspase-3 antibody was obtained from R & D systems, p53 antibody (clone DO-1) was purchased from Oncogene Science, Cambridge, MA, U.S.A. Cox-1 (SC-1752), Cox-2 (SC-1745) and anti-bax (SC7480) antibodies were obtained from Santa Cruz, Santa Cruz, California, U.S.A. Ki-67 (Ki-S5), anti-bcl-2 (124), Streptavidin-AP, Streptavidin-HRP, normal rabbit serum, normal swine serum, rabbit anti-mouse (biotinylated) and swine anti-rabbit (biotinylated) were obtained from DAKO Ltd, Ely, UK. BSA and Tris ultra pure were obtained from ICN Biomedicals, Basingstoke, UK.

Formaldehyde, methanol, NaCl, MgCl₂, citric acid monolydrate and haematoxylin were obtained from Fisher Scientific, Loughborough, UK; and DPX mountant and Aquamount from BDH Poole, UK.
Annexin-V-FITC was purchased from Alexis Biochemicals, Nottingham UK.

RNasin, AMV-RT, and Taq polymerase were purchased from Promega; NBT/BCIP tablets and dNTPs were purchased from Roche. 100bp ladder was purchased from Invitrogen.

All other chemicals were purchased from Sigma-Aldrich.

2.1.3 Colonic Tissue

Human colonic tissue was obtained during biopsy or surgical resection from patients undergoing partial or total colectomy; each patient gave informed consent. All tissues were obtained at the Leicester Royal Infirmary, Leicester, UK. Control (normal) colonic tissue was obtained from a disease free area of the colon (as determined by a gastrointestinal pathologist). UC grade was determined independently by histological examination of tissue. Tissue from untreated UC was obtained from patients at initial diagnosis. Patients undergoing therapy with any form of NSAID, other than 5-ASA, were excluded from the study. Ethical approval for the study was obtained from the University Hospitals of Leicester NHS Trust.

2.2 Methods

2.2.1 Cell Culture

HT-29, HCT-15, SW480 and CaCo-2 cells were maintained in 80 cm² culture flasks containing 18 ml culture medium (DMEM plus 10% FCS, RPMI 1640 plus 20% FCS, L-15 plus 10% FCS, 1% L-glutamine, 1% non-essential amino acids
(NEAA) or DMEM plus 10% FCS, 1% L-glutamine, 1%NEAA respectively), cells were maintained at 37°C, 5% CO₂. Cell lines were passaged at approximately 90% confluency; cells were washed in HBSS, and exposed to trypsin/EDTA until detached from culture flasks. Trypsin was inactivated by washing twice in the appropriate culture medium. Cells were pelleted by centrifugation, resuspended and cell number determined by trypan blue counts. Cells were then diluted in the appropriate culture medium to give the correct cell density.

2.2.2 Determination of Cell Number
100 µl 0.4% trypan blue was added to 100 µl cell suspension, mixed well and left at RT for 5 min. 50 µl was pipetted onto a haemocytometer and cells counted in the two central squares to obtain an average cell number.

2.2.3 Treatment of Cells with Indomethacin/5-ASA
Cells were grown to 90% confluency, then trypsinised and a cell count performed as previously described (see sections 2.2.1 and 2.2.2). Cells were pelleted by centrifugation and resuspended in the appropriate medium to give a final concentration of 5 x 10⁵ cells per ml. 2 ml of cell suspension was added to each well of a six well plate. Cells were incubated at 37°C 5% CO₂ for 24 hours. Next day, 2 µl of indomethacin/5-ASA (0.2/0.1 M in DMSO) was added, this gave a final concentration of 0.1% DMSO for each well, and resulted in final concentrations of 200 µM or 100 µM indomethacin/5-ASA. Negative controls contained 0.1% DMSO only. Cells were incubated at 37°C 5% CO₂ for 24, 48 or 72 hours.
Following treatment, medium was transferred into a centrifuge tube, cells were washed twice with 1 ml HBSS, and this was pooled with the removed medium. Cells were trypsinised and added to the floating cell population. Cell counts were carried out to determine cell number. Cells were spun onto slides using the cytospin, as described below (section 2.2.4). Apoptosis in treatment groups was determined by immunohistochemistry, using the anti-caspase-3 antibody (see section 2.2.7). The number of positively stained cells in four quadrants of each cytospin were counted and an average score obtained.

2.2.4 Cytospin Preparation

Cell culture medium was removed and retained; attached cells were washed twice in HBSS then detached by adding 3 ml trypsin/EDTA. Attached and floating cell populations were pooled and cell number determined using trypan blue. Cytospins were prepared using the Shandon Cytospin™ preparation system according to manufacturers instructions. Briefly, cells were centrifuged, the supernatent was removed and the pellet washed twice in HBSS, before resuspending in HBSS to gave a final concentration of 2 x 10^6 cells/ml. 500 μl cell suspension was used per saline treated slide and cells spun at 1500 rpm for 5 min on low acceleration in the cytospin. Slides were fixed immediately in ice-cold methanol at -20°C for 30 min then washed twice in PBS. Slides were then air-dried and stored at -80°C until use.

2.2.5 Cytoblock preparation

Cytoblocks were prepared using the Shandon Cytoblock™ Cell Block Preparation system according to manufacturers instructions. Briefly, attached and floating cell populations from the test flask were pooled and were centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and cells fixed by resuspending in 4%
formaldehyde at RT for 30 min. Cells were re-pelleted by centrifugation at 1000 rpm for 5 min and the supernatant removed, leaving the pellet as dry as possible. The pellet was resuspended in 4 drops of reagent 2 and mixed thoroughly by vortexing. 3 drops of reagent 1 were added to the centre of the well of the cytoblock cassette, which was then assembled and placed in the Cytospin. The cell suspension mix was placed in the funnel and blocks were Cytospun at 1500 rpm for 5 min at low acceleration. The funnels were discarded and 1 drop of reagent 1 was placed in the centre of the Cytoblock well. Cassettes were then closed and fixed in 70% ethanol at RT. The resulting cell button was embedded in paraffin wax and sectioned at a thickness of 5 μM.

2.2.6 Colonic Tissue Sample Processing

Biopsy specimens were fixed in 10% formal saline and processed (sometimes with heat). Surgical samples were fixed in 10% formal saline for 48 hours before processing. Samples were embedded in paraffin wax. 4 μm sections were cut and dried at 37°C for 24 hours before use.

2.2.7 Immunohistochemistry

Paraffin embedded sections were dewaxed and rehydrated. If 3, 3-diaminobenzidine (DAB) was ultimately the chromogen used, sections were incubated in 3% H2O2 for 10 min, then washed for 10 min in tap water before proceeding. Antigen retrieval was carried out by immersion in 10 mM citrate buffer solution (4.2g citric acid monohydrate in 2L UP H2O, adjusted to pH 6 with 5 M NaOH), and heating in a microwave oven at 750 W for 15 min. Slides were cooled, and then washed 5 x 5 min in TBS. Cytospin slides were defrosted and washed in TBS before use. 100 μl of the appropriate blocking solution was added
Sections were blocked for 10 min, then excess solution drained and 100 μl primary antibody added. The anti-caspase-3, and p53 antibodies were added at 1:1000, Cox-1, Cox-2, Ki-67, anti-Bcl2 and anti-Bax were added at 1:100. All sections were incubated at 4°C for 18 hours.

The following day, sections were washed 5 x 5 min in TBS then incubated for 30 min at RT with 100 μl secondary antibody, (rabbit anti-mouse (biotinylated) 1:400 for Bcl-2, Bax, p53 and Ki-67; swine anti-goat (biotinylated) 1:400 for Cox-1 and Cox-2; swine anti-rabbit (biotinylated) 1:1000 for capsase-3). Slides were then washed 5 x 5 min in TBS and incubated with 100 μl streptavidin-HRP (DAB to be used as chromogen) or 100 μl streptavidin-AP, (NBT/BCIP to be used as chromogen), (982 μl TBS 9 μl A 9 μl B, made up 30 min before use) for 30 min at RT.

2.2.7.1 Colour Development - DAB

Sections were washed 5 x 5 min in TBS, then 100 μl DAB added and slides incubated until colour development could be seen (approximately 5 min). Sections were then rinsed in tap water for 5 min, and counterstained by dipping shortly in haematoxylin. After a further 5 min rinse, sections were dehydrated and mounted using DPX medium.

2.2.7.2 Colour Development - NBT/BCIP

Sections were rinsed 5 x 5 min in TBS followed by 1 x 5 min in UP H₂O. NBT/BCIP was made up by dissolving one NBT/BCIP tablet in 10 ml UP H₂O. 200 μl working solution was added to each slide, sections were coverslipped and
incubated at RT in the dark. Colour development was monitored using a light microscope. Sections were then washed for 5 min in tap water, and mounted with Aquamount.

Negative controls were carried out for all experiments, where the primary antibody was omitted. All other steps were carried out as above.

Results were analysed by light microscopy.

2.2.8 Preparation of Cells for Flow Cytometry

2.2.8.1 Detection of Cell Viability

Cells, seeded at 0.1 x 10⁶/ml were treated with indomethacin or 5-ASA for 24, 48 or 72 hours as previously described. At the appropriate time point culture medium was removed, and centrifuged to pellet out floating cells. These cells were washed in 1 ml PBS then resuspended in 190 μl Annexin-V binding buffer (10 mM HEPES; 150 mM NaCl; 1 mM MgCl₂; 5 mM KCl; 1.8 mM CaCl₂). 1 μl of Annexin-V-FITC and 10 μl propidium iodide (PI) (2 μg/ml sterile H₂O) were then added and samples incubated for 10 min at RT in the dark. 800 μl Annexin-V binding buffer was then added and samples centrifuged at 1300 rpm for 5 min. Pellets were resuspended in 400 μl PBS and kept on ice.

Meanwhile, apoptosis was detected in adhered cells using a method first described by van Engeland et al (1996). This method involves staining cells with Annexin-V-FITC before detaching from culture flasks. Attached cells were washed twice with HBSS before adding 1 ml Annexin V binding buffer. 2 μl Annexin-V-FITC and 40 μl PI (2 μg/ml sterile H₂O) were then added and samples incubated at 37°C for 10
min in the dark. Following this, the Annexin-V binding buffer was removed and cells washed twice with a further 1 ml Annexin-V binding buffer. Cells were then detached from wells using 1 ml trypsin/EDTA, this action was stopped by the addition of 5 ml culture medium. Cells were pelleted by centrifugation at 1000 rpm for 5 min and then washed in 1 ml PBS. Finally cells were resuspended in 600 µl Annexin V binding buffer and pooled with the floating cell populations before analysis by flow cytometry.

Cell death was detected by passing cells through an argon laser beam. After gating out cell debris on the basis of forward and side scatter properties, emissions from fluorescent dyes were analysed on a logarithmic scale, and plotted on a dual parameter histogram to detect and distinguish between Annexin-V, and PI positive cells. The histogram was divided into four quadrants to separate viable, apoptotic, necrotic, and dual stained cells (figure 2.1). The percentage of cells within each quadrant was recorded.

2.2.8.2. Cell Cycle Studies

Cells, seeded at 0.1 x 10^6/ml were treated with indomethacin or 5-ASA for 24, 48 or 72 hours as previously described. At the appropriate time point, culture medium was removed and discarded, cells were detached from culture flasks with trypsin/EDTA and this reaction stopped by the addition of 5 ml culture medium. Cells were pelleted by centrifugation at 1000 rpm for 5 min; cells were then washed in HBSS before resuspending in 500 µl HBSS. Cells were fixed by the addition of 2 ml ice-cold ethanol (70%) and left at 4°C overnight. Next day cells were washed once in PBS before resuspending in 800 µl PBS. 100 µl RNase (10 mg/ml RNase; 10 mM Tris-HCl; 15 mM NaCl) was added to each sample to remove RNA, and
DNA was stained by the addition of 100 μl PI (50 μg/ml). Samples were incubated at 37°C for 30 min before analysis by flow cytometry.

Position of cells within the cell cycle was determined by detection of DNA content which corresponded to level of PI fluorescence. Cells were passed through an argon laser beam and cell debris excluded from analysis on the basis of forward and side scatter properties. Red fluorescence (PI) intensity was detected and plotted on a linear scale. Peaks corresponding to the sub G₀ (apoptotic) G₀/G₁ and G₂/M populations were gated and the number of cells within each gate calculated (figure 2.2).

2.2.9 Production of Cell Lysates

Cells were detached from culture flasks by treatment with trypsin/EDTA. Cells were then centrifuged at 1000 rpm for 5 min and resuspended in lysis binding buffer (100 mM Tris pH 8.0; 500 mM LiCl; 10 mM EDTA pH 8.0; 1% SDS, 5 mM DTT) to give a final concentration of 5 x 10⁶ cells/ml. This was divided into 100 μl aliquots and 5 μl 1 mg/ml proteinase K/DEPC was added to each aliquot. Cell lysates were then incubated at 37°C for 1 hour before storage at -20°C until use.

2.2.10 Extraction of mRNA using Dynabeads

Cell lysates were defrosted and DNA sheared by repeat pipetting through a 21G followed by a 25G needle. Samples were microfuged briefly to reduce frothing. Dynabeads (suspended in PBS) were isolated using a Dynal MPC and the supernatant was discarded. Beads were washed once, then resuspended in lysis binding buffer. 30 μl conditioned dynabeads were added to each sheared lysate and
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Figure 2.2 Analysis of cell-cycle distribution by flow cytometry

Cells were fixed in ethanol and DNA stained by propidium iodide (PI). DNA content of cells was determined by flow cytometry. Cells were passed through an argon laser beam and cell debris excluded from the study on the basis of forward and side scatter properties. Red fluorescence (produced by PI) was detected (FL3). Fluorescence intensity corresponds to DNA content and position within the cell cycle. Peaks corresponding to cells in the $G_0/G_1$ (D); and $G_2/M$ (E) phases of the cell cycle were gated and the number of cells within each gate determined. A sub-diploid peak, corresponding to apoptotic cells was also seen (C).
Figure 2.1 Analysis of cell death by flow cytometry.

Cells were dual stained with Annexin-V-FITC and propidium iodide (PI) in order to detect apoptosis and necrosis respectively. Cells were analysed by flow cytometry by passing through an argon laser beam. Cell size was determined by forward and side scatter properties, and cell debris (grey dots) removed from further analysis by gating out (A). Emission of PI and Annexin-V-FITC fluorescence was detected and plotted on a logarithmic scale (B); FL1 indicates the level of Annexin-V fluorescence and FL3 indicates PI fluorescence. Histograms were divided into four quadrants representing viable (E3), apoptotic (E4), necrotic (E1), and dual stained cells (E2). Percentage of cells in each quadrant was recorded for further analysis.
samples left for 5 min at RT to allow mRNA to bind. Following this, beads were pelleted in the Dynal MPC, then washed twice in 60 µl of wash buffer with LiDS (10 mM Tris pH 8.0; 0.15 M LiCl; 1 mM EDTA; 0.1% SDS), and twice with wash buffer without LiDS (10 mM Tris pH 8.0; 0.15 M LiCl; 1 mM EDTA) before being resuspended in 30 µl DEPC H₂O. Samples were stored at 4°C until use.

2.2.1.1 Production of cDNA

Reaction mixes containing 10 µl mRNA, 5 µl 5 x AMV buffer (250 mM Tris-HCl pH 8.3; 250 mM KCl; 50 mM MgCl₂; 50 mM DTT; 2.5 mM spermidine), 2.5 µl 10 mM dNTPs/DEPC, 0.62 µl Rnasin (25U), 0.5 µl AMV-RT (5U) and DEPC H₂O (to give a total of 25 µl) were incubated at 42°C for 1 hour using a Hybaid PCR machine. Reverse transcription reactions were carried out on the same day as mRNA extraction. cDNA was then stored at 4°C until use.

2.2.1.2 Polymerase chain reaction

Primers were designed to amplify target sequence using the Primer 3 primer design program. GAPDH, Cox-1, Cox-2, Bax, Bcl-XL and PPAR α, γ and δ were amplified using oligonucleotide primer pairs (table 2.1). The target sequence was amplified using PCR in reaction volumes of 50 µl containing 42 µl sterile UP H₂O, 5 µl 10 x AJ buffer (45 mM Tris pH 8.8; 11 mM (NH₄)₂SO₄; 4.5 mM MgCl₂; 200 µM dNTPs; 110 µg/ml BSA; 6.7 mM β-mercaptoethanol; 4.4 µM EDTA pH 8), 1 µl forward primer (10 pm/µl), 1 µl reverse primer (10pm/µl), and 1 µl cDNA. Samples were overlaid with 50 µl light paraffin oil. 2 µl (1 unit) Taq polymerase (5U Taq polymerase diluted 1:10 in 1 x AJ buffer) was added following the initial denaturation step. Amplification was carried out in a Hybaid thermal cycler using the following reaction conditions; 98°C 30 sec initial denaturation step, annealing
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Sequence and position of forward primer (5'-3')</th>
<th>Sequence and position of reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AF261085</td>
<td>AGAACATCATCCCTGCCTC (714-732)</td>
<td>GCCAAATTCGTTGTCATACC (1060-1041)</td>
</tr>
<tr>
<td>Bax</td>
<td>L22473</td>
<td>TCTGACGGCAACTTCAACTG (301-320)</td>
<td>AACCACCTGGTCTTTGGAT (472-454)</td>
</tr>
<tr>
<td>Bcl-Xl</td>
<td>Z23115</td>
<td>CTTGGATGGCCACTTACCTG (637-656)</td>
<td>TGTCTGGTCATTTCGACTG (843-824)</td>
</tr>
<tr>
<td>Cox-1</td>
<td>AF440204</td>
<td>TCTGGCTACGTAGACGACAAAC (13600-13619)</td>
<td>CTCAGCTGCTGCACGTACTC (16437-16418)</td>
</tr>
<tr>
<td>Cox-2</td>
<td>M90100</td>
<td>ATGGACCAGAGCAGGCAGAT (1409-1428)</td>
<td>CACAGCATCGATGTCCCAT (1558-1539)</td>
</tr>
<tr>
<td>PPARα</td>
<td>NM_005036</td>
<td>TTGGTGCTGTATCTATTTGC (1345-1365)</td>
<td>TCCGACTCCGTCCTTCTTGAT (1570-1551)</td>
</tr>
<tr>
<td>PPARγ</td>
<td>D83233</td>
<td>CATAAGTCCTCCCAGCTGA (911-930)</td>
<td>TCTGTGATCTCTGCACAGC (1138-1119)</td>
</tr>
<tr>
<td>PPARδ</td>
<td>AF246302</td>
<td>AGCCAGTACAAAAACCACAGGT (183-202)</td>
<td>TTCAACAACTGCTTCCACAC (565-546)</td>
</tr>
</tbody>
</table>

Table 2.1 Details of primers used in these studies
at 72°C for 30 sec, followed by 4 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 sec and amplification at 72°C for 30 sec, this was followed by a further 25 (for GAPDH primers), 30 (for Cox-1, Bax, Bcl-X\textsubscript{L}, PPAR\textgreek{a} and PPAR\textgreek{y} primers) or 35 (for Cox-2 and PPAR\textgreek{y} primers) cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and amplification at 72°C for 30 sec. 18 \( \mu \)l PCR product was mixed with 2 \( \mu \)l loading buffer and analysed alongside 20 \( \mu \)l 100bp ladder by electrophoresis at 80 volts on a 3% agarose gel (3 gm agarose dissolved in 1 x TEA buffer (40 mM Tris; 1 mM EDTA; 0.001% v/v glacial acetic acid); 5 \( \mu \)l/100 ml ethidium bromide was added to the gel to allow visualisation of the products). Bands were detected by UV light, gel pictures photographed and images stored for further analysis. Level of gene expression was quantified by analysis of band intensity using Scion Image.

2.2.13 Statistical Analysis of Results

All statistical analysis and calculations were performed using MINITAB software.

2.2.13.1 Analysis of Immunohistochemical Studies

The mean average and standard deviation values were calculated from the raw data, (cell counts, \( N=4 \) for each time point and treatment; apoptosis counts, \( N=8 \) for each time point and treatment) Data was analysed using a paired t-test.

2.2.13.2 Cell Turnover in UC

The mean and standard error of the mean (SEM) were calculated from the raw data obtained from each disease stage (severe UC \( N=17 \); quiescent UC \( N=12 \); pre-treated \( N=7 \) 5-ASA treated \( N=14 \)). The data obtained in these studies was non-continuous, therefore analysis was carried out using the Mann-Whitney U-test.
2.2.13.3 *Analysis of Flow Cytometry*

Mean values obtained from 4 experiments were calculated and these values used to determine the mean and SEM values. Data is normally distributed and statistical analysis was therefore carried out using a t-test to determine if there was significant difference between paired control and treated groups.

2.2.13.4 *Analysis of Gene Expression*

Levels of gene expression were determined by analysis of band intensity using Scion Image, following RT-PCR amplification. Data was analysed using the paired t-test to compare gene expression in control samples, with 5-ASA or indomethacin treated cell lines.
Chapter 3.

*Apoptosis and Proliferation Levels in Normal, Inflamed and Neoplastic Colonic Mucosa; The Effects of Disease Severity and 5-ASA Treatment on Cell Turnover in Ulcerative Colitis*
3.1 Introduction

Cellular apoptosis and proliferation play a crucial role in the maintenance of colonic homeostasis. Colonic epithelial cell turnover is high. Stem cells located in the crypt base undergo differentiation and migrate up crypt walls and to the luminal surface where they are lost in a process that takes only three to eight days. Apoptosis is thought to play an important role in the removal of cells at the luminal surface, and is therefore a key mechanism in controlling crypt epithelial cell number.

Due to the balance of cellular proliferation and apoptosis and the rapid cell turnover which is found within the colon, it is easy to see how alterations in the levels of either of these growth-controlling mechanisms could result in the onset of various pathological disorders. Colonic neoplasia is an obvious end point which may result from an imbalance of cell growth and death rates, with an increase in proliferation and/or a decrease in apoptosis levels being likely causes. Previous research however has suggested that apoptosis may actually be increased in colonic adenomas and carcinomas (Hawkins et al, 1997) suggesting that increases in the rate of epithelial cell proliferation may be a contributing factor in the development of a malignant phenotype (see section 1.4.5.2).

Alterations in expression levels of the Bcl-2 family of genes will result in a change to the susceptibility of cells towards apoptosis or survival. The anti-apoptotic gene Bcl-2, and the pro-apoptotic gene Bax are known to be capable of forming homodimers. An increase in the cellular ratio of Bcl-2:Bax results in a decrease in the susceptibility of cells to undergo apoptosis (and conversely an increase in Bax:Bcl-2 ratio favours apoptosis) (see section 1.4.4). Mutations or altered expression levels in either of these two genes within the colonic...
mucosa may result in inappropriate cell turnover, and (depending on the gene affected), could lead to cell accumulation or epithelial breakdown.

In addition to a role in neoplasia, alterations in the balance of cell growth and death may be of importance in IBD. During idiopathic IBD there is a breakdown and loss of the colonic mucosa. Research carried out by others has suggested that an increase in apoptosis levels may, at least in part, be responsible for this (Iwamoto et al, 1996; Hagiwara et al, 2002). A reduction in the level of apoptosis of T cells within the lamina propria may contribute to the chronic inflammatory state that is associated with CD (Itoh et al, 2000). Alterations in the level of apoptosis within the lamina propria in UC are less clear, and conflicting reports exist (Strater et al, 1997; Bu et al 2001) (see section 1.4.5.3).

5-ASA containing compounds are the drugs of choice in the treatment of UC despite the fact that the mechanistic pathways behind their efficiency at treating this disorder are unknown (see section 1.8). As UC is a chronic disorder treatment is often continued for many years. Whilst UC is known to pre-dispose to colonic cancer, there is some evidence to suggest that the 5-ASA compounds may be chemopreventive (MacGregor et al, 2000; Narisawa et al, 2002). Due to the structural similarities between 5-ASA and aspirin (a NSAID known to induce apoptosis in malignant cells), the possibility exists that 5-ASA compounds may also induce apoptosis within colonic cells, this suggests a possible mechanistic pathway for chemoprevention. Experiments carried out in patients suffering from colorectal cancer have suggested that 5-ASA increases apoptosis in malignant cells (Bus et al, 1999). The effects of 5-ASA on cell proliferation are less clear and conflicting reports exist; whilst Bus et al (1999) did not see any effects of 5-ASA on proliferation in patients given mesalazine (the market
name for 5-ASA), MacGregor et al (2000) reported an inhibition of proliferation in 5-ASA treated cell lines.

The studies described in this chapter were designed, firstly, to examine the levels of apoptosis and proliferation in normal colonic mucosa and in various colonic disorders. Progressive stages of neoplasia were examined to investigate the possible role of apoptosis and proliferation in the development of malignancy. Changes in Bcl-2 and Bax expression were analysed by immunohistochemistry to determine if mutations in either of these genes may play a role in the neoplastic process. The role of cell turnover in UC was investigated by comparing apoptosis and proliferation levels in severe and quiescent UC. Secondly, studies were designed to investigate the effects of 5-ASA treatment on apoptosis and proliferation levels within the colonic mucosa to provide further insight into the mechanistic pathways behind the ability of this drug to resolve UC, as well as its chemopreventive properties.
3.2 Analysis of Apoptosis in Normal, Neoplastic and Inflamed Colonic Mucosa

Apoptotic cells were detected immunohistochemically by the anti-caspase-3 (active) antibody, in formalin-fixed paraffin embedded tissue sections taken from normal, hyperplastic polyp, adenoma and adenocarcinoma colonic mucosa, which had been obtained with patient consent following biopsy or colectomy.

Apoptosis levels within the epithelium of normal tissue were very low (<1%) and these apoptotic cells were confined to the luminal surface (figure 3.1A). No apoptosis was seen in crypt epithelium. Apoptosis within the lamina propria was low (grade 1 (for grading scale see figures 3.4 and 3.5)) and was localised near the luminal surface (figure 3.1B). Apoptosis was not increased in epithelial cells within hyperplastic polyps, however within the lamina propria apoptosis levels were increased (grade 3) and these apoptotic cells were found distributed further down the lamina propria (figure 3.1C).

Adenomas showed greatly increased levels of apoptosis in comparison to normal tissue. Apoptotic cells appeared focally within the tissue, with some glands containing up to 40% apoptotic cells, and others showing only 2% apoptosis (figure 3.1D), overall approximately 10% of epithelial cells were apoptotic. Similarly adenocarcinomas showed an average of 10% apoptosis (n=7 SEM=1.53), however, unlike adenomas, this staining was uniform throughout the sections (figure 3.1E).

An increase in the number of apoptotic lymphoid cells within the lamina propria was seen in inflamed tissue, however as with normal tissue, these apoptotic cells were generally located
near to the luminal surface (3.1F). In contrast to this, apoptosis within the lamina propria of UC tissue, whilst less dense, was generally distributed evenly throughout the lamina propria (3.1G) furthermore intraepithelial lymphocytes undergoing apoptosis were seen invading crypts. Unlike in normal tissue, the presence of apoptotic epithelial cells was seen within colonic crypts, however, levels of apoptosis within crypt cells were low (1-2%) (figure 3.1H).

3.3 Proliferation in Normal and Neoplastic and UC Colonic Mucosa

Proliferation levels in normal, adenocarcinoma, and UC tissue were analysed by immunohistochemistry using the Ki-67 antibody.

Proliferation in epithelial cells of normal tissue was confined to the crypt base; within this area approximately 43% of cells were stained (depending on the crypt) (n=10 SEM=3). No proliferation was seen in epithelial cells in the upper half of crypts or on the luminal surface. Proliferation within the lamina propria was low (grade 1 (see figure 3.4 for grading scale)) (figure 3.2A). Proliferation and apoptotic levels of lymphocyte cells within the lamina propria were approximately equal, indicating balanced turnover of these cells in normal tissue.

In colonic mucosa obtained from adenomas and adenocarcinomas, proliferation was seen in approximately 60% of epithelial cells (n=9 SEM=2.81) (figure 3.2B). This staining appeared in a uniform pattern throughout the colonic epithelium, rather than being confined to the lower half of the crypt as was seen in normal tissue, these results indicated a deregulation in the normal control of epithelial cell growth in colonic adenomas.
Apoptosis was detected using the anti-caspase-3 (active antibody) in formalin fixed paraffin-embedded colonic mucosa tissue, which had been obtained at biopsy or colectomy. Positive cells were visualised by colour reaction with NBT/BCIP (B, C, D, F, G, H) or DAB (A, E). Normal tissue (obtained from an unaffected region within the colon) had low levels of epithelial apoptosis confined to the luminal surface (A), apoptotic cells were also found in the lamina propria, again these were seen proximal to the gut lumen (B). Apoptosis was increased in the lamina propria of hyperplastic polyps, but no increase in epithelial apoptosis was observed (C). Neoplastic tissue demonstrated increased levels of epithelial apoptosis, which appeared focally in adenomas (D) and uniformly in carcinoma (E). Inflamed tissue had increased levels of apoptosis within the lamina propria, which was located near the luminal surface (F), within UC this increase in apoptosis appeared throughout the lamina propria (G), the presence of apoptotic cells within colonic crypts was frequently seen in cases of severe UC (H).
Figure 3.1 Immunohistochemical detection of apoptosis in normal, inflamed and neoplastic human colonic mucosa obtained from patients diagnosed with colonic inflammation, active ulcerative colitis, hyperplasia or neoplastic conditions.
Figure 3.2 Immunohistochemical detection of proliferation in normal, neoplastic and inflamed human colonic mucosa obtained from patients diagnosed with colonic neoplasia or ulcerative colitis.

Proliferating cells were detected by immunohistochemistry using the Ki-67 antibody in formalin fixed paraffin-embedded colonic mucosa. Positive cells were visualised by colour staining with NBT/BCIP. Within normal tissue (obtained from an unaffected region of the colon) proliferating cells were mainly located in the lower half of epithelial crypts and proliferation within the lamina propria was low (A). Adenomas and adenocarcinomas showed greatly increased rates of proliferation, which was seen throughout epithelial cells (B). Rates of cell proliferation both in epithelial cells, and within the lamina propria varied between cases of UC. Proliferating cells were seen higher up crypt walls than in normal tissue, and proliferation was generally increased in the lamina propria (C). Proliferating intraepithelial lymphocytes were seen invading crypts, and within crypt abscesses (D).
Proliferation levels of epithelial cells in UC varied widely between cases; levels of epithelial proliferation varied from an average of 10% of cells within the colonic crypts in one patient, to 80% of crypt cells in another. Unlike in normal tissue, proliferating cells were seen extending high up the walls of the crypts, whilst in most cases proliferation was concentrated in the lower half of the crypt region, proliferating cells were detected at lower numbers extending to the top of crypts (figure 3.2C). Surface epithelium was frequently lost so it was not possible to determine apoptosis or proliferation levels within this area.

Proliferation of lymphocyte cells within the lamina propria of UC tissue also varied, with levels from near normal (grade 1) to grade 4 seen. These proliferating cells were distributed evenly throughout the lamina propria and were seen infiltrating crypt epithelium, as well as in crypt abscesses figure 3.2(D). Levels of proliferation seen in both the lamina propria and epithelium of UC tissue appeared to correspond to the level of tissue damage (assessed by histology) and as a result of these studies, further work was carried out to examine the correlation of apoptosis and proliferation levels with UC disease severity (see section 3.5).

3.4 Bcl-2 and Bax Expression in Normal, Inflamed and Neoplastic Colonic Mucosa.

Immunohistochemical analysis of Bcl-2 and Bax protein levels was carried out in corresponding normal, neoplastic and inflamed colonic mucosa. Within normal tissue Bax expression was confined to the luminal surface and the top third of crypts, with all cells staining positive on the luminal surface (figure 3.3A). Bax positive lymphocyte cells were seen scattered throughout the lamina propria, where approximately 50% of cells stained positively for Bax. The levels of Bax protein within the lamina propria did not significantly alter in cases of severe UC (figure 3.3B). No staining was seen in epithelial cells, however
much of the surface epithelium was lost in many of the sections examined. Epithelial cells in adenocarcinomas were all found to be uniformly Bax positive (figure 3.3D).

Bcl-2 immunoreactivity of normal epithelium was very low, and was localised to a few cells in the base of crypts, however in the lamina propria, Bcl-2 staining was high (approximately 50%), and was found to be distributed throughout the thickness of the lamina propria (figure 3.3E). A similar pattern of Bcl-2 staining was seen in inflamed tissues, however the number of positive cells was generally less than in normal colonic mucosa. Intraepithelial lymphocyte cells were seen in low numbers in some colonic crypts (figure 3.3F). The pattern of Bcl-2 expression in UC was similar to that seen in inflamed tissue, however large aggregates of Bcl-2 positive lymphocyte cells (lymphoid follicles) were seen (figure 3.3G). The number of Bcl-2 positive intraepithelial lymphocytes was greatly increased in UC tissue in comparison to inflamed tissue. Furthermore occasional Bcl-2 positive epithelial cells were seen scattered in colonic crypts, these were generally found in the bottom half of crypts, and were not localised to stem cells in crypt bases, as had been seen in normal tissue (figure 3.3G).

Bcl-2 immunoreactivity was increased in hyperplastic polyps in comparison to normal controls. Staining was seen in approximately 3% (n=5, SEM=1.53) of epithelial cells and was located in random positions within the colonic crypts (data not shown). Finally, all colonic adenomas and adenocarcinomas examined were found to be Bcl-2 negative (figure 3.3H); surrounding lamina propria contained levels of Bcl-2 positive cells similar to those seen in inflamed tissue.
Bax (A, B, C, D) and Bcl-2 (E, F, G, H) protein was detected by immunohistochemistry in formalin fixed paraffin-embedded colonic mucosa. Positive cells were visualised by colour staining with NBT/BCIP (except B where detection is with DAB). Within normal tissue (obtained from an unaffected region of the colon) Bax was confined to the luminal surface, whilst Bcl-2 was detected in stem cells located at the base of crypts (A and E respectively). Bcl-2 staining was high within normal lamina propria (E). The distribution and level of Bax protein did not alter significantly in UC (B). Bcl-2 staining was decreased in the lamina propria in UC in comparison to normal tissue, however large collections of inflammatory cells that were diffusely positive were seen, as were intraepithelial lymphocytes and occasional Bcl-2 positive epithelial cells within crypts (G). Intraepithelial lymphocytes were also seen in inflamed tissue (F), although to a lesser extent than in UC. Colonic adenocarcinomas were diffusely positive for Bax (D, C = negative control), but failed to show any Bcl-2 staining (H).
Figure 3.3 Immunohistochemical detection of Bax and Bcl-2 in normal, inflamed and neoplastic human colonic mucosa obtained from patients diagnosed with colonic inflammation, active ulcerative colitis or colonic neoplasia
3.5 Comparison of Apoptosis and Proliferation Levels with UC Severity

Experiments were designed to examine and compare the levels of apoptosis and proliferation with UC disease severity. Apoptosis and proliferation were detected in UC tissue biopsies by the anti-caspase-3 (active) and Ki-67 antibodies respectively. Levels of staining within the lamina propria, intraepithelial lymphocytes and epithelial cells were graded as described in figures 3.4 and 3.5. Results were then compared with UC severity (severe or quiescent) as determined independently by a clinician.

Apoptosis levels within the lamina propria were significantly lower in quiescent, than in severe UC (figure 3.6) (average grades of 1.09 and 2.00 for quiescent and severe disease respectively; p=0.0155). Levels of apoptotic intraepithelial lymphocytes were also significantly lower in quiescent disease (quiescent = grade 1.00, severe = grade 1.61; p=0.0229) (although this may be due to a general reduction in the number of these cells in quiescent, compared to severe disease). Whilst the number of apoptotic cells within the epithelium appeared to be reduced in quiescent UC, this was not found to be statistically significant.

Comparison of proliferation levels with disease severity showed a significant decrease in the level of proliferation, both in the lamina propria (p=0.0002), and in crypt epithelial cells (p<0.0000) in quiescent (grades 1.5 and 2.43 in the lamina propria and crypt epithelium respectively), when compared to severe UC (grades 3.06 and 5.88 in lamina propria and crypt epithelium respectively) (figure 3.7). Furthermore, proliferating cells were generally located lower down in the crypt in quiescent disease, than in severe UC.
3.6 Comparison of Apoptosis and Proliferation Levels in Pre-Treated and 5-ASA Treated UC

To gain further insight into the mechanisms underlying the mode of action, and the possible chemopreventive effects of the 5-ASA compounds, experiments were designed to analyse and compare apoptosis and proliferation levels in UC colonic mucosa at initial biopsy (before treatment), and following treatment with 5-ASA (or a 5-ASA containing compound). Apoptosis and proliferation levels were determined by immunohistochemistry with the anti-caspase-3 and Ki-67 antibodies respectively. Proliferation was scored as shown in figure 3.4, whilst blinded to the treatment group.

Results showed a significant decrease in epithelial apoptosis following treatment with 5-ASA in comparison to untreated controls (grades 0.247 and 1.33 in 5-ASA and untreated samples respectively; p=0.02) (figure 3.8). Whilst results suggested a small increase in the apoptosis levels of cells within the lamina propria and of intraepithelial lymphocytes, this was not found to be statistically significant.

Treatment with 5-ASA was not found to significantly effect proliferation levels in either epithelial cells or within the lamina propria (figure 3.9). Results however, suggest a possible increase in the levels of proliferation within crypts following 5-ASA treatment; a large variability was seen in epithelial proliferation levels in pre-treated samples and this could have effected the significance of the results obtained.
In summary, results presented in this chapter demonstrate that apoptosis in normal colonic mucosa is confined to the luminal surface, whilst proliferation is found towards the crypt base. Both apoptosis and proliferation within the lamina propria of normal tissue is low.

Colonic adenomas and carcinomas showed an increase in the level of apoptosis, which was seen focally and uniformly throughout crypts, in adenomas and adenocarcinomas respectively. Similarly proliferation levels were increased in comparison to normal controls and proliferating cells were found throughout colonic crypts. These results corresponded to an increase in the Bax:Bcl-2 ratio. All colonic adenocarcinomas analysed demonstrated uniform Bax staining, coupled to a total absence of Bcl-2 protein.

Apoptosis within the lamina propria was increased in inflamed colonic mucosa, with apoptotic cells generally located near to the luminal surface. Apoptosis was seen throughout the lamina propria in UC and increased with disease severity. Proliferation was increased in both epithelial cells and within the lamina propria of severe UC in comparison to quiescent disease, and normal controls.

Finally, analysis of apoptosis and proliferation levels in colonic mucosa obtained from patients that have undergone 5-ASA therapy demonstrated a decrease in epithelial apoptosis following treatment, in comparison to pre-treated controls.
Grading of Apoptosis and Proliferation in Lamina Propria

<table>
<thead>
<tr>
<th>% Positive cells</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>0</td>
</tr>
<tr>
<td>1-5</td>
<td>1</td>
</tr>
<tr>
<td>6-10</td>
<td>2</td>
</tr>
<tr>
<td>11-20</td>
<td>3</td>
</tr>
<tr>
<td>21+</td>
<td>4</td>
</tr>
</tbody>
</table>

Grading of Apoptosis and Proliferation in Intraepithelial Lymphocytes

<table>
<thead>
<tr>
<th>% Crypt area showing positively-stained infiltrating cells</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
</tr>
<tr>
<td>1-5</td>
<td>1</td>
</tr>
<tr>
<td>6-10</td>
<td>2</td>
</tr>
<tr>
<td>11-20</td>
<td>3</td>
</tr>
<tr>
<td>21+</td>
<td>4</td>
</tr>
</tbody>
</table>

Grading of Apoptosis in Epithelial cells

Grading corresponds to percentage of positive cells
e.g. 2% cells positive = grade 2

Grading of Proliferation in Epithelial Cells (seen in crypts only)

<table>
<thead>
<tr>
<th>% Crypt Positive</th>
<th>Grade</th>
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<tr>
<td>1-10</td>
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<td>61-70</td>
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</tr>
<tr>
<td>71-80</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 3.4 Scoring system used for analysis of immunohistochemical detection of apoptosis and proliferation.
Figure 3.5 Scoring system for immunohistochemical detection of apoptosis and proliferation in lamina propria

Immunohistochemistry was carried out as described in materials and methods. Sections were scored on a grade of 0 to 4 based on the percentage of positive cells within the lamina propria. 

<1% cells positive = grade 0; 1-5% cells positive = grade 1 (A); 6-10% cells positive = grade 2 (B); 11-20% cells positive = grade 3 (C); 21+% cells positive = grade 4 (D).
Figure 3.6 Comparison of apoptosis levels in severe and quiescent UC

Apoptosis was detected by immunohistochemistry using the anti-caspase-3 (active) antibody as described in materials and methods. The number of apoptotic cells in the lamina propria (LP), intraepithelial lymphocytes (IL) and epithelial cells were counted and apoptotic scores given as shown in figure 3.4. Quiescent UC had significantly less apoptotic cells within the lamina propria, and lower levels of apoptotic intraepithelial lymphocytes, in comparison to severe disease (*p<0.05). Severe UC N=17; Quiescent UC N=12; bars indicated SEM.
**Figure 3.7 Comparison of proliferation levels in severe and quiescent UC**

Apoptosis was detected by immunohistochemistry using the anti-caspase-3 (active) antibody as described in materials and methods. The number of proliferating cells in the lamina propria (LP), and crypt epithelium were counted and proliferation scores given as shown in figure 3.4. Quiescent UC had significantly less proliferating cells within the lamina propria and epithelium in comparison to severe disease (**p<0.01). Severe UC N=17; Quiescent UC N=12; bars indicate SEM.
Figure 3.8 Comparison of apoptosis levels in untreated and 5-ASA treated UC

Apoptosis was detected by immunohistochemistry using the anti-caspase-3 (active) antibody as described in materials and methods. The number of apoptotic cells in the lamina propria, intraepithelial lymphocytes (IL) and epithelial cells were counted and apoptotic scores given as shown in figure 3.4. Treatment with 5-ASA significantly decreased the number of apoptotic epithelial cells (*p<0.05). Pre-treated UC N=7; 5-ASA treated UC N=14; bars indicate SEM.
Figure 3.9 Comparison of proliferation levels in untreated and 5-ASA treated UC

Apoptosis was detected by immunohistochemistry using the anti-caspase-3 (active) antibody as described in materials and methods. The number of proliferating cells in the lamina propria, and crypt epithelium were counted and proliferation scores given as shown in figure 3.4. 5-ASA treatment did not significantly alter proliferation levels in the lamina propria or epithelium. Pre-treated UC N=7; 5-ASA treated UC N=14; bars indicate SEM.
3.7 Discussion

The studies described in this chapter were designed to analyse the levels of cellular apoptosis and proliferation within the colonic mucosa of normal, inflamed and neoplastic tissue. A comparison of cell turnover in severe and quiescent UC was carried out to determine if changes in either cell growth or death rates may, at least in part, be responsible for the chronic inflammatory state, and the breakdown of surface epithelium that occurs in this disorder. Finally, the effects of patient therapy with 5-ASA on apoptosis and proliferation levels within the colonic mucosa were examined.

Apoptosis within the normal colon was very low (<1%) and was confined to the luminal surface, whilst proliferation was confined to the base region of the crypts. These results are in agreement with the theory that colonic stem cells migrate up from crypt bases, differentiate into functional epithelial cells, and are eventually lost at the luminal surface (Liu et al, 1999). This theory is further supported by the expression of the pro-apoptotic gene Bax, which was seen exclusively at the luminal surface and within the top third of epithelial crypts; and the expression of the anti-apoptotic gene Bcl-2, which was only seen in stem cells located at the very base of crypts.

Colonic adenomas and carcinomas demonstrated greatly increased rates of apoptosis. Within adenomas, apoptotic cells appeared focally, suggesting the existence of differing clones of cells within the adenoma. In carcinomas however, apoptosis was seen uniformly at a rate of about 10-15% throughout the malignant tissue. This suggests that colonic carcinoma cells undergo an increased rate of apoptosis, in comparison to that seen in normal tissue, and inhibition of this process may not be underlying in the pathogenesis of colonic malignancy.
These findings are in agreement with increased rates of apoptosis found in neoplastic tissue by others (Flohil et al, 1996; Hawkins et al, 1997; Sinicrope et al, 1995). Proliferation rates were also found to be increased in neoplastic tissue (50-70% in comparison to 30-50% seen in normal tissue), and proliferating cells were seen throughout crypts, rather than being confined to stem cells located in the crypt base (as is seen in normal tissue). Whilst an increase in epithelial cell apoptosis was observed in colonic cancer, the apoptotic rate remained low in comparison to the high proliferation rates observed. These observations suggest that deregulation of cellular proliferation, resulting in increased growth rates, and proliferation of inappropriate cells may contribute to the neoplastic phenotype.

The apoptotic rate found in tumour tissue in this study (10-15%) is marginally higher than that reported by others (0.2-7%, (Sinicrope et al, 1995)), this is probably due to methodological differences; previous studies have detected apoptosis using the in situ end labelling (ISEL) method which detects the DNA fragmentation which occurs in the late stages of apoptosis. In contrast to this, the anti-caspase 3 (active) antibody detects cells from an earlier stage of apoptosis (activation of caspase-3 precedes DNA fragmentation) and is therefore likely to detect a wider time frame of the apoptotic process, leading to an apparently increased result.

In contrast to these studies, Bedi et al (1995) described high levels of apoptosis in normal tissue (20-30%), which progressively decreased in adenomas and carcinomas. This study by Bedi et al was carried out in frozen tissue and single cell suspensions (cultured from epithelial biopsies). It is probable that within single cell suspensions those cells obtained from neoplastic tissue would be less sensitive to loss of cell to cell contacts than would normal
cells; this could account for the increased rate of apoptosis in normal, compared to tumour cells that was reported.

The increase in apoptosis seen in neoplastic tissue corresponded to an increase in Bax, and decrease in Bcl-2 expression. All adenocarcinomas analysed in these studies showed diffuse Bax staining within epithelial cells, whilst no Bcl-2 staining was seen. Studies carried out by others have demonstrated decreased expression of Bcl-2 in colonic carcinomas (Hawkins et al, 1997; Flohil et al, 1996), however some adenomas have been reported to be diffusely positive for Bcl-2 (Valassiadou et al, 1997; Flohil et al 1996). It would be useful to have gained information regarding the grading of tumour stage in the neoplastic tissue that was used in experiments, as it is possible that over expression of Bcl-2 (and hence resistance to apoptosis) may play a role in the development of early adenomas, but is lost during later stages of neoplasia. Bcl-2 expression may then have not been detected, if all sections used in these studies were obtained from later stage tumours. The elevation, and diffuse staining pattern of Bax seen in adenocarcinomas in these studies, has also been described by others (Hirose et al, 1997, Maurer et al 1998) and mutations, resulting in the over expression of Bax is known to be associated with colorectal cancers with a microsatellite mutator phenotype (MMP+).

Unlike in neoplastic tissue, the level of apoptosis in hyperplastic-polyp lesions (which are non-neoplastic) was not increased in comparison to normal tissue. Immunohistochemical detection of Bcl-2 in these lesions showed increased Bcl-2 expression in comparison to normal controls. Furthermore these positively stained cells were detected higher up the walls of the crypts, and were not confined to stem cells in the crypt base. These results suggest that
the inhibition of apoptosis through increased Bcl-2 expression may play a role in the pathogenesis of hyperplastic polyps within the colon.

Within inflamed tissue, apoptosis within the lamina propria was greatly increased in comparison to normal controls; these apoptotic cells were located near to the luminal surface. These cells are likely to play an important role in protection of the colonic mucosa from invading micro-organisms that may be present in the gut, this would explain why the majority of activity seen in inflammation is located near to the luminal surface.

In UC however, apoptotic cells were found distributed throughout the lamina propria and apoptotic intraepithelial lymphocytes were seen throughout crypts. An increase in the proliferation of lymphocytes within the lamina propria was also seen and proliferation rates were higher than the rate of apoptosis detected in adjacently cut sections. These results demonstrate an increase in the number of inflammatory cells within the lamina propria, which exists within the mucosa of UC tissue. No change in the levels of Bax expression within the lamina propria of UC tissue was observed in comparison to normal controls. Bcl-2 levels within the lamina propria however, appeared to be lower than those found in normal tissue, however large areas of Bcl-2 staining lymphocyte aggregates were seen. Together these results suggest that within active UC there is an increase in the proliferation of inflammatory cells within the lamina propria, whilst Bcl-2 expression is decreased (increasing the Bax:Bcl-2 ratio). Therefore apoptosis levels are increased in UC in comparison to normal controls, this level of apoptosis is not high enough to balance the proliferation rate, and therefore over-proliferation of inflammatory cells within the lamina propria may be responsible in part for the maintenance of the chronic state of inflammation. The observation that rates of
proliferation and apoptosis of lymphocytes balance in normal tissue and quiescent disease further support this theory.

Work carried out by others (Bu et al, 2001) has suggested that levels of T cell apoptosis within the lamina propria are decreased in patients with UC in comparison to normal controls and that, as T cell apoptosis is an important mechanism in switching off an immune response, inability of these T cells to undergo apoptosis will result in an inappropriately prolonged response to environmental antigens (exposure to which is a possible trigger for the onset of UC). The resistance of T cells to apoptosis has been found to be associated with an increase in the Bcl-2:Bax ratio in CD (but not in UC) (Itoh et al, 2001). Results obtained in the studies presented in this chapter found increased levels of apoptosis within the lamina propria in UC in comparison to normal tissue, however this does not provide any specific information about the cell types undergoing apoptosis. In order to gain more insight into cell types undergoing apoptosis or proliferation in UC dual staining experiments could be carried out.

Apoptosis of epithelial cells was increased in UC tissue, although levels were still low. Occasional apoptotic cells were seen in epithelial crypts, indicating loss of normal cell death regulation. Proliferation rates within UC colonic crypts were increased (average of 60% compared to 10-30% seen in normal tissue) The proliferation rate observed in epithelial cells was far higher than the rate of apoptosis, however, much of the surface epithelium was lost, and whilst it is possible that the apoptotic process may have been responsible for this, these cells would not be detected as they will quickly be shed into the gut lumen. Furthermore, detection of apoptotic cells depended on detection of activated caspase-3, In vivo apoptotic cells are cleared by phagocytosis and secondary necrosis and these cells will not be detected by immunohistochemistry, therefore only a proportion of apoptotic cells that are within a
certain phase of apoptosis will be visualised, and the overall apoptotic rate may be under-represented.

Proliferation rates of epithelial cells were much higher in severe in comparison to quiescent disease. Apoptosis also decreased in quiescent disease (although this was not found to be statistically significant). Together these results suggest that active severe UC results in increased cell-turnover; over a long period of time, this increase in cell turn-over will predispose to the accumulation of genetic mutations, which may lead to neoplasia; hence the predisposition of UC sufferers to the development of colorectal cancer.

Various studies suggest that 5-ASA treatment may decrease the risk of developing colorectal cancer (Macgregor et al, 2000, Narisawa et al 2002). The mechanisms underlying possible chemopreventive effects, and the resolution of UC by 5-ASA are not fully understood. Studies presented in this chapter have shown that treatment of UC sufferers with 5-ASA, or 5-ASA containing compounds, results in decreased epithelial apoptosis. This reduction of epithelial apoptosis may in part explain why 5-ASA is useful in the treatment of UC, as it may facilitate the regeneration of the epithelial barrier, which is frequently lost in severe UC. Results obtained suggested a possible increase in apoptosis within the lamina propria however this was not found to be statistically significant. The lack of significance of this result may be due to the large variance seen between samples in the pre-treatment group. The number of pre-treated samples was low (n=7), and furthermore, whilst the majority of samples were paired (i.e. pre-treated samples were obtained from the same patient as treated samples), due to problems with ethical approval it was not possible to gain access to all archived pre-treated biopsy material. This resulted in some pre-treated samples being obtained prospectively, and it was not possible to pair these samples with treated colonic biopsies. Carrying out
experiments in a larger sample group, and ensuring all pre- and post-treated samples are paired could improve the study; in addition this would eliminate possible variation in results due to the innate variability in colonic cell turnover between individuals.

Proliferation levels within the lamina propria did not significantly alter following 5-ASA treatment (therefore if apoptosis levels are increased in response to 5-ASA treatment, this may help to reduce, and resolve the chronic state of inflammation). Proliferation levels within epithelial cells appeared to increase, but this was not found to be statistically significant; again this could be due to small pre-treated sample size and differing disease severity.

Overall, the results obtained in these studies suggest that 5-ASA may help to resolve severe UC by the regeneration of the epithelial barrier, this should, in turn, reduce the number of invading micro-organisms and reduce the chronically active immune response. Whilst there is no obvious effects of 5-ASA on cell turn-over that may explain its possible chemopreventive properties (e.g. increased epithelial apoptosis or decreased proliferation), the resolution of UC from severe to quiescent disease results in a significant reduction in epithelial cell proliferation. Therefore if 5-ASA works to ultimately resolve severe UC, this in turn will result in a reduction in cell proliferation within colonic crypts, reducing the propensity for mutations to occur, and therefore reducing the risk of neoplasia developing.
Chapter 4.

The Effects of Indomethacin and 5-ASA on Cell Viability in Human Colonic Cell Lines.
4.1 Introduction

Various lines of investigation, including epidemiological, in vivo and in vitro studies have provided strong evidence to suggest that NSAIDS are chemopreventive within the colon, however the mechanisms underlying these chemopreventive properties remain unknown. Whilst it is generally agreed that NSAIDS are capable of reducing tumour growth through the induction of apoptosis in malignant colorectal epithelial cells (reviewed by Elder and Paraskeva, 1999), the mechanism through which apoptosis is induced remains unknown. The effects of NSAIDs on cell proliferation are less clear and conflicting reports exist (Pasricha et al, 1995; Sawaoka et al, 1998) (see section 1.6).

Both Cox-2 and prostaglandin levels have been shown to be frequently elevated in human colonic neoplasia, and the fact that the NSAIDs are thought to exert their antipyretic and anti-inflammatory effects largely through the inhibition of prostaglandin synthesis due to inhibition of Cox raises the possibility that the chemopreventive properties of the NSAIDs may also be due to Cox inhibition. Several studies however suggest that this is not the case; NSAIDs have been shown to be capable of inducing apoptosis in cell lines that lack any Cox transcripts and furthermore, the concentration of NSAID needed to inhibit tumour cell growth does not correlate with the concentration required to inhibit prostaglandin biosynthesis. The evidence for and against Cox-dependent mechanisms of chemoprevention by NSAIDs is reviewed by Shiff and Rigas (1999).
The tumour-suppressor gene p53 is an important regulatory gene in the progression of cell cycle and the induction of apoptosis. Activation of p53 leads to the transcription of genes that inhibit cell cycle and trigger a check-point in the G₁ phase of the cell-cycle, and if DNA is irreparably damaged, p53 is capable of triggering apoptosis. As both a block in the G₁ phase cell-cycle, and induction of apoptosis have been observed following NSAID treatment of colonic cells, it is possible that p53 may be an important mediator in the mechanism of NSAID induced chemoprevention. However experiments carried out by others have suggested that NSAIDs are capable of inducing apoptosis in p53 mutant cell lines (Shiff et al, 1995; Piazza et al, 1997).

Drugs containing 5-ASA are commonly used for the treatment and control of ulcerative colitis. The structural similarities between 5-ASA and the NSAID aspirin (5-acetylsalicylic acid) raise the possibility that 5-ASA may also possess chemopreventive properties, previous in vitro and in vivo studies carried out by other workers have suggested that 5-ASA treatment may induce apoptosis in malignant colorectal cells (Bus et al, 1999). The effects of 5-ASA on proliferation are unclear and conflicting reports regarding the ability of 5-ASA to inhibit proliferation exist (Bus et al, 1999; MacGregor et al, 2000) (see section 1.8).

Various NSAIDs have been shown to induce cell-cycle arrest in G₁. Published data suggests that 5-ASA may also induce a cell-cycle block, however whilst MacGregor et al (2000) report a block in the G₂/G₁ phase of the cell cycle (as seen with indomethacin), Reinacher-Schick et al (2003) have reported that 5-ASA treatment of colonic cells
resulted in accumulation of cells in G2/M. The effects of 5-ASA on the cell-cycle remain unclear.

To summarise, studies to date suggest that 5-ASA may possess chemopreventive properties, however, the majority of studies have been carried out in animal models and very few studies exist examining the effects of 5-ASA on human colonic tissue in vivo and in human colonic cell lines. Furthermore, the mechanisms by which both 5-ASA and NSAIDs induce apoptosis are as yet unknown.

The studies described in this chapter were designed to investigate and compare the effects of the NSAID indomethacin and 5-ASA on human colonic cell lines, in particular with regard to cell viability, cell cycle distribution and the induction of apoptosis. Four cell lines were chosen, these being CaCo-2, HCT-15, HT-29 and SW480. These cell lines are derived from human colonic adenocarcinomas, are well characterised in existing literature. The cell lines represent various tumour grades, and differ in their expression of Cox-1 and -2 as detailed in table 4.1. The aim of these studies was firstly to examine and compare the effects of indomethacin and 5-ASA treatment on human colonic carcinoma cell lines, to ascertain whether 5-ASA treatment results in inhibition of tumour cell growth and if like indomethacin, 5-ASA is capable of inducing apoptosis in malignant cells; and secondly how any observed chemopreventive properties 5-ASA may have compare with the conventional NSAID indomethacin. Furthermore, chemopreventive ability was compared with tumour grade, Cox-1 and -2 and p53 expression to examine whether chemoprevention correlates with malignant grade, or is dependant on Cox.
expression or the presence of wild-type p53 for inhibition of cell cycle and induction of apoptosis.

Finally the effects of indomethacin and 5-ASA treatment on cell-cycle were analysed by flow cytometry, firstly to confirm reports by others that NSAIDs cause G₁ arrest, and secondly to further clarify the effects of 5-ASA on the cell-cycle of malignant colonocytes.

Cell lines were treated with 5-ASA or indomethacin at 24 hourly intervals for 72 hours. This treatment was designed to represent the daily dosing that a patient may be exposed to. Previous work carried out by others has demonstrated an inhibition of cell growth and the induction of apoptosis in colonic cell lines following treatment with 100-200 μM NSAIDs (Piazza et al, 1997; Shiff et al, 1995; Hanif et al, 1996), and therefore for these experiments, indomethacin and 5-ASA were added at 100 or 200 μM. The concentration of 5-ASA and its metabolites that exist within the colonic mucosa during treatment for UC is unclear, and mucosal concentrations of 5-ASA differ in the rectum to those found in the sigmoid colon (Naganuma et al, 2001). Reported values for mucosal tissue concentrations include 6.6 μg/g and 12 ng/ml (Naganuma et al, 2001 and Hussain et al, 2001 respectively). 200 μM is equivalent to 30.6 μg/ml, which whilst higher than reported mucosal concentrations within rectal tissue, is similar to mucosal concentration within the sigmoid colon (22.2 μg/g) (Naganuma et al, 2001). It was decided to use 5-ASA concentrations of 100 and 200 μM in order to directly compare the efficacy of 5-ASA with indomethacin with regards to chemopreventive potential.
4.2 Characterisation of Cell Lines

The human colonic cell lines CaCo-2, HCT-15, HT-29 and SW480 have been frequently used as *in vitro* models of malignant human colonic cells within the literature. Whilst the genetic status of each cell line (table 1) is widely accepted, by their very nature rapidly dividing cell lines, which are derived from mutated human cells, will be prone to further mutation. Studies were therefore designed to ascertain the p53 and Cox status of these cell lines.

Cells were grown in the appropriate culture medium for 48 hours. At this point, cells were detached from culture flasks with the use of trypsin, before being cyto-spun onto glass slides, or lysed and used to make cDNA as described in materials and methods (see sections 2.2.3 and 2.6.9-2.6.11). P53 and Cox status were confirmed by immunohistochemistry, and the levels of Cox-1 and -2 mRNA transcripts examined through RT-PCR.

Figure 4.1 shows the results of immunohistochemical detection of p53 in the four cell lines. P53 protein was detected in the SW480 cell line, however this cell line demonstrated cytoplasmic staining only, indicating a mutated p53 phenotype. Nuclear P53 protein was detected in the HCT-15 cell line. No expression of p53 was seen in CaCo-2 or HT-29 cells.
Figures 4.2-4.3 and figure 4.4 show the immunohistochemical detection, and PCR amplification respectively, of the Cox-1 and -2 isoforms. Immunohistochemistry detected low levels of Cox-1 in both Caco-2 and HT-29 cell lines (figure 4.2 A, C). No immunoreactivity was seen with the Cox-1 antibody in the HCT-15 cell line (figure 4.2 B) however, unexpectedly, low levels of Cox-1 were detected in the SW480 cell line (figure 4.2 D). The presence of Cox-1 in the SW480 cell line was further supported by the presence of a faint band following PCR amplification (figure 4.4). Experiments were repeated for further clarification, and results obtained were identical to the above observations, indicating a true result.

Cox-1 mRNA was detected in the HT-29 cell line, which was in agreement with results obtained by immunohistochemistry. The SW480 and HCT-15 cell line appeared to produce low levels of Cox-1 mRNA (although no Cox-1 protein was detected in the HCT-15 cells); whilst no Cox-1 mRNA was detected in the CaCo-2 cell line, despite the fact that immunohistochemistry had given positive results (table 4.2).

Examination of Cox-2 mRNA and protein levels suggest that Cox-2 is expressed and translated in both the HT-29 and CaCo-2 cell lines (table 4.2 and figures 4.3 and 4.4), with levels being greatest in CaCo-2 cells. No Cox-2 expression was seen in either HCT-15 or SW480 cells; these findings are in agreement with the published data.
### Table 4.1: Features of the human colonic cell lines used in these studies as characterised within existing literature.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Differentiation</th>
<th>Tumour Grade</th>
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<tbody>
<tr>
<td>HT-29</td>
<td>Good I</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CaCo-2</td>
<td>Good II</td>
<td>+ truncated</td>
<td>+ (low)</td>
</tr>
<tr>
<td>SW480</td>
<td>Poor III-IV</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCT-15</td>
<td>Poor II</td>
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Figure 4.1 Immunohistochemical detection of p53 in human colonic cell lines.

Immunohistochemistry, using a p53 antibody, was carried out in cytopsins. Positively stained cells were observed in the HCT-15 (B) and SW480 (D) cell lines. No p53 immunoreactivity was seen in CaCo-2 (A) or HT-29 cells (C).
Figure 4.2 Immumohistochemical detection of Cox-1 in human colonic cell lines.

Immunohistochemistry, using a Cox-1 antibody, was carried out in cytopsins. Positive cells were detected by DAB staining. CaCo-2 (A), HT-29 (C) and SW480 (D) all showed Cox-1 immunoreactivity. The HCT-15 cell line was negative for Cox-1 (B). Figures E and F represent no primary antibody negative controls for the HT-29 and HCT-15 cell lines respectively.
Figure 4.3 Immunohistochemical detection of Cox-2 in human colonic cell lines

Immunohistochemistry, using a Cox-2 antibody was carried out in cytospins. CaCo-2 cells were seen to contain high levels of Cox-2 protein (A), Ht-29 cells were also positive for Cox-2 (C). Neither the HCT-15 (B) nor SW480 (D) cell lines showed COX-2 immunoreactivity. Figures E and F represent no primary antibody negative controls for the HT-29 and HCT-15 cell lines respectively.
Figure 4.4 Expression of Cox-1 (top row) and Cox-2 (bottom row) in human colonic cell lines.

HCT-15, HT-29, CaCo-2 and SW480 cells were grown in culture flasks using the appropriate medium for 72 hours before harvesting. Cells were lysed and mRNA extracted. cDNA was produced and PCR carried out with primers designed to detect Cox-1 or Cox-2 as described in materials and methods. PCR products were separated by electrophoresis in a 3% agarose gel. M indicates 100bp DNA marker, +/- indicates plus and minus RT reactions respectively, N indicates cDNA negative controls. Results indicate low Cox-1 expression in HCT-15 and SW480 cell lines. A higher level of Cox-1 expression is seen in HT-29 cells. Cox-2 is expressed in both HT-29 and CaCo-2 cells, however expression is much higher in the CaCo-2 cell line. The presence of the 650 bp band, seen along the bottom row, may indicate amplification of the Cox-2 gene, or the presence of non-spliced mRNA.
### Table 4.2 Expression and translation of p53, Cox-1 and Cox-2 in human colonic cell lines used in these studies.

P53, Cox-1 and Cox-2 protein was detected by immunohistochemistry in human cell line cytospins. Cox-1 and Cox-2 mRNA was detected in cell lines by RT-PCR. + indicates weak signal following immunohistochemistry, or low band density following agarose gel electrophoresis and detection of RT-PCR product. ++ indicates strong signal following immunohistochemistry, or the presence of a dense band following agarose gel electrophoresis and detection of RT-PCR product.
4.3 Does Indomethacin or 5-ASA Treatment Reduce Cell Viability, or Induce Apoptosis in Human Colonic Cell Lines?

Studies were designed to examine the effects of the NSAID indomethacin, and 5-ASA on the human colonic cell lines. Cell viability and apoptosis was measured and compared using both immunohistochemistry and flow cytometry. Cells undergoing apoptosis or proliferation were detected using antibodies raised against cleaved caspase-3 or the nuclear antigen Ki-67 respectively. Flow cytometry was used to distinguish between viable, apoptotic and necrotic cells through the use of propidium iodide and FITC-conjugated annexin V to detect necrotic and apoptotic cells respectively.

The methodology is detailed in chapter 2 (see sections 2.2.3-2.2.4 and 2.2.7-2.2.8). Briefly, cells were seeded in 75ml flasks and incubated at 37°C, 5% CO₂ for 24 hours to allow attachment of cells to the flask. Following this, cells were treated with 100 or 200 μM indomethacin or 5-ASA dissolved in DMSO; controls were treated with DMSO only. Treatment was repeated at 24 hourly intervals for the duration of the study. Cells were harvested at 24, 48 and 72 hours, cell numbers were determined and cells either spun onto slides for immunohistochemical analysis, or analysed by flow cytometry.
4.3.1 **Immunohistochemical Analysis of Apoptosis in Indomethacin and 5-ASA Treated Cell Lines.**

At the appropriate time point, cells were detached from flasks by trypsinisation, cell numbers were determined by haemocytometer counts and immunohistochemistry carried out using the anti-caspase-3 (active) antibody to detect apoptosis.

The data obtained from these experiments is shown in figures 4.5 to 4.8. Indomethacin was found to reduce cell number, with a concurrent increase in apoptosis in a dose dependant manner. This effect was seen in all four cell lines tested (table 4.3; page 142). A decrease in cell number was apparent from the 48 hour time point in the SW480 and HCT-15 cell lines, but was not apparent until the 72 hour time point in CaCo-2 and HT-29 cells; however an increase in apoptosis was seen in CaCo-2 and HT-29 cells at 48 hours following treatment with 200 μM indomethacin.

Treatment of cell lines with 200 μM indomethacin resulted in an approximate 3 fold increase in apoptosis levels in comparison to the DMSO controls, this effect was less in the CaCo-2 cell line, despite the fact that indomethacin treatment in this cell line resulted in the greatest decrease in cell number of all cell lines examined.

Treatment of cells with 5-ASA did not appear to increase apoptosis in any of the cell lines, and no effect was seen on cell number in the CaCo-2 and HCT-15 cells. Results however, did suggest a decrease in cell number at 72 hours following treatment with 100
or 200 μM 5-ASA in the SW480 cell line (figure 4.8), and at 72 hours following treatment with 200 μM 5-ASA in the HT-29 cell line (figure 4.7).

To summarise, the results of the immunohistochemical studies showed that indomethacin treatment of colonic cell lines results in a significant decrease in the number of viable cells and an increase in the number of cells undergoing apoptosis, these effects were dose dependent. 5-ASA treatment was not seen to induce apoptosis, although a decrease in cell number was seen in the HT-29 and SW480 cell lines at the 72 hour time point.

In order to further test the above data, an alternative approach was used to examine the effects of indomethacin and 5-ASA treatment on the same four cell lines. For these experiments, cell were treated with the appropriate drug in the exact same way as previously described, flow cytometry was then used to ascertain cell viability and apoptosis levels.
Figure 4.5 Effect of indomethacin and 5-ASA treatment on cell number and apoptosis in the CaCo-2 cell line

Cells were treated with 100 or 200 μM indomethacin/5-ASA at 24 hourly doses for up to 72 hours as described in materials and methods. At the appropriate time point, cells were harvested. Cell counts were carried out, before cells were spun onto slides. Apoptosis was detected by immunohistochemistry, using the caspase-3 antibody. Values represent mean cell number or apoptotic rate calculated from four experiments. Bars indicate SEM. Indomethacin, but not 5-ASA treatment, significantly reduced cell number and increased apoptosis of CaCo-2 cells in a dose dependant manner (*P<0.05; ** P<0.01).
Figure 4.6 Effect of indomethacin and 5-ASA treatment on cell number and apoptosis in the HCT-15 cell line

Cells were treated with 100 or 200 μM indomethacin/5-ASA at 24 hourly doses for up to 72 hours as described in materials and methods. At the appropriate time point, cells were harvested. Cell counts were carried out, before cells were spun onto slides. Apoptosis was detected by immunohistochemistry, using the caspase-3 antibody. Values represent mean cell number or apoptotic rate calculated from four experiments. Bars indicate SEM. Indomethacin reduced cell number and increased apoptosis of HCT-15 cells in a dose dependant manner (*P<0.05; ** P<0.01).
Figure 4.7 Effect of indomethacin and 5-ASA treatment on cell number and apoptosis in the HT-29-2 cell line

Cells were treated with 100 or 200 μM indomethacin/5-ASA at 24 hourly doses for up to 72 hours as described in materials and methods. At the appropriate time point, cells were harvested. Cell counts were carried out, before cells were spun onto slides. Apoptosis was detected by immunohistochemistry, using the caspase-3 antibody. Values represent mean cell number or apoptotic rate calculated from four experiments. Bars indicate SEM. Indomethacin reduced cell number and increased apoptosis of HT-29 cells in a dose dependent manner. A decrease in cell number is seen following 200μM 5-ASA treatment at the 72-hour time point (*P<0.05; **P<0.01).
Figure 4.8 Effect of indomethacin and 5-ASA treatment on cell number and apoptosis in the SW480 cell line

Cells were treated with 100 or 200 μM indomethacin/5-ASA at 24 hourly doses for up to 72 hours as described in materials and methods. At the appropriate time point, cells were harvested. Cell counts were carried out, before cells were spun onto slides. Apoptosis was detected by immunohistochemistry, using the caspase-3 antibody. Values represent mean cell number or apoptotic rate calculated from four experiments. Bars indicate SEM. Indomethacin, but not 5-ASA treatment, reduced cell number and increased apoptosis of SW480 cells in a dose dependant manner (*P<0.05; **P<0.01).
4.3.2 FACs Analysis of Cell Viability, Apoptosis and Necrosis in Indomethacin and 5-ASA Treated Cell Lines.

Treated cells were labelled with propidium iodide and FITC conjugated annexin-V in order to label necrotic and apoptotic cells respectively and then sorted by flow cytometric analysis. Viable (unlabelled), apoptotic and necrotic cell populations were represented graphically as distinct populations as shown in figure 4.9, the percentage of cells in each population was determined and these figures used to represent cell viability, apoptosis and necrosis for each treatment time and group expressed as a ratio of the control (figures 4.10 to 4.13).

Treatment of cells with indomethacin resulted firstly, in increased annexin-V-FITC fluorescence (figure 4.9 B, D, and F) and secondly, in the appearance of a cell population with decreased forward and side scatter, indicating cell shrinkage (figure 4.9 A, C, and E); both of these observations are indicative of the induction of apoptosis.

A significant decrease in cell viability, together with an increase in the percentage of cells undergoing apoptosis is seen at the 48 and 72 hour time points in CaCo-2 and HT-29 cell lines (figures 4.10 and 4.12 respectively) and at 72 hours in the SW480 cell line (figure 4.13). Whilst the data obtained from HCT-15 cells suggests a decrease in cell viability and increase in apoptosis at similar level to that seen in the HT-29 cell line, this is not statistically significant (table 4.3). This is probably due to the large variation in values obtained between experiments.
The SW480 cell line appeared to be the most sensitive to indomethacin treatment, with an approximate 26-fold increase in apoptosis 72 hours after initial treatment with 200 μM indomethacin. At the same time-point, CaCo-2 cells show an approximate 5 to 6-fold increase in apoptosis whilst HCT-15 and HT-29 cells both show a 2 to 3-fold increase.

No significant changes in cell viability or apoptosis were seen in response to treatment with 5-ASA; although results suggested a decrease in viability, coupled to an increase in apoptosis in the HCT-15 cell line, this diminishes with time and was not statistically significant.

In summary, the data obtained from flow cytometric analysis of the effects of indomethacin and 5-ASA on human colonic cell lines demonstrates the ability of indomethacin to significantly reduce cell viability and induce apoptosis in human colonic cell lines, this occurs in a dose dependant fashion; These observations are in agreement with the data obtained by immunohistochemical analysis (section 4.3.1). No significant effects are seen following treatment with 5-ASA.
Figure 4.9 FACS analysis of cell viability.

SW480 Cells were treated with 100 or 200μM indomethacin or 5-ASA at 24 hourly intervals. At 72 hours cells were harvested, and dual stained with annexin-V-FITC and propidium iodide in order to detect apoptosis and necrosis respectively. Cell debris was excluded from analysis (A, C, E, G) and cell viability was determined by FACS analysis, level of annexin-V FITC staining is shown on the X-axis (FL1 log) and propidium iodide on the Y-axis (FL3 log) (B, D, F, H). Graphs are divided into quadrants to separate viable (E3), apoptotic (E4) and necrotic (E1) cell populations for analysis. Quadrant E2 contains dual stained cells, which are undergoing secondary necrosis. Indomethacin treatment decreased cell viability and increased apoptosis in a dose dependant manner. No effect was seen following 5-ASA treatment.
Figure 4.10 Comparison of the effects of 5-ASA and indomethacin on CaCo-2 cells.

Cells were treated with 200 or 100 μM 5-ASA/indomethacin at 24 hourly intervals. Cells were harvested at 24, 48 and 72 hours following initial treatment and cell viability, apoptosis and necrosis levels determined by flow cytometry as described in materials and methods. Graphs show the ratio of viable, apoptotic and necrotic cells as compared to untreated (DMSO only) controls. Time points (hours) are indicated on the X-axis. Results show mean values obtained from four analyses. Bars indicate SEM. Indomethacin treatment resulted in a dose dependant decrease in cell viability, accompanied by an increase in the level of apoptosis (*P<0.05 **P<0.01). No effect was seen following 5-ASA treatment.
Figure 4.11 Comparison of the effects of 5-ASA and indomethacin on HCT-15 cells.

Cells were treated with 200 or 100 μM 5-ASA/indomethacin at 24 hourly intervals. Cells were harvested at 24, 48 and 72 hours following initial treatment and cell viability, apoptosis and necrosis levels determined by flow cytometry as described in materials and methods. Graphs show the ratio of viable, apoptotic and necrotic cells as compared to untreated (DMSO only) controls. Time points (hours) are indicated on the X-axis. Results show mean values obtained from four analyses. Bars indicate SEM. Indomethacin treatment resulted in a dose dependant decrease in cell viability, accompanied by an increase in the level of apoptosis (*P<0.05). No effect was seen following 5-ASA treatment.
Figure 4.12. Comparison of the effects of 5-ASA and indomethacin on HT-29 cells.

Cells were treated with 200 or 100 µM 5-ASA/indomethacin at 24 hourly intervals. Cells were harvested at 24, 48 and 72 hours following initial treatment and cell viability, apoptosis and necrosis levels determined by flow cytometry as described in materials and methods. Graphs show the ratio of viable, apoptotic and necrotic cells as compared to untreated (DMSO only) controls. Time points (hours) are indicated on the X-axis. Results show mean values obtained from four analyses. Bars indicate SEM. Indomethacin treatment resulted in a dose dependant decrease in cell viability, accompanied by an increase in the level of apoptosis (*P<0.05, **P<0.01). No effect was seen following 5-ASA treatment.
Figure 4.13. Comparison of the effects of 5-ASA and Indomethacin on SW480 cells.

Cells were treated with 200 or 100 μM 5-ASA/indomethacin at 24 hourly intervals. Cells were harvested at 24, 48 and 72 hours following initial treatment and cell viability, apoptosis and necrosis levels determined by flow cytometry as described in materials and methods. Graphs show the ratio of viable, apoptotic and necrotic cells as compared to untreated (DMSO only) controls. Time points (hours) are indicated on the x-axis. Results show mean values obtained from four analyses. Bars indicate SEM. Indomethacin treatment resulted in a dose dependant decrease in cell viability, accompanied by an increase in the level of apoptosis (*P<0.05, **P<0.01). No effect was seen following 5-ASA treatment.
4.3.3 Effects of Indomethacin and 5-ASA Treatment on Cell Cycle.

To explore the mechanism responsible for the reduction in cell viability that was seen following indomethacin treatment, experiments were designed to examine the effects of indomethacin treatment on cell cycle distribution. The effects of 5-ASA were also examined to see if inhibition of cell cycle may explain the possible reduction in cell number seen in the HT-29, SW480 (section 4.3.1) and HCT-15 cell lines (section 4.3.2). Cells were treated with indomethacin or 5-ASA at 24 hourly intervals for 24, 48 or 72 hours. At the appropriate time, cells were harvested and fixed in ethanol; DNA was stained with propidium iodide and the DNA content of cells determined by FACS analysis.

Indomethacin treatment of colonic cell lines was found to decrease the proportion of cells in the S and G₂/M phases of the cell cycle with a simultaneous increase in the proportion of cells in G₀/G₁ (figure 4.14; table 4.3). The appearance of a novel sub-diploid peak representing cells undergoing apoptosis was seen following treatment with 200 μM indomethacin in all cell lines (although to a lesser extent in the HT-29 and CaCo-2 cells (data not shown)).

Results showed the HT-29 cell line to be most sensitive to alterations in cell cycle phase distribution in response to indomethacin treatment (figure 4.17). A highly significant decrease in the proportion of cells in the S and G₂/M phases, accompanied by an equally
significant increase in the proportion of cells in G\textsubscript{0}/G\textsubscript{1}, was seen from 48 hours following treatment with either 100 or 200 \textmu M indomethacin.

Other cell lines appeared to be less sensitive to the effects of indomethacin on the cell cycle. A decrease in the S and G\textsubscript{2}/M phases was seen from 48 hours following treatment with 100 or 200 \textmu M indomethacin in the HCT-15 and CaCo-2 cell lines (figures 4.16 and 4.15 respectively), the fact that this has not been found to be statistically significant is probably due to variability between experiments. This was accompanied by a significant increase in the number of cells in G\textsubscript{0}/G\textsubscript{1} in the CaCo-2 cell line, which was not seen with HCT-15 cells, however, the reduction in the proportion of HCT-15 cells in G\textsubscript{0}/G\textsubscript{1} seen at 72 hours was accompanied by the appearance of the novel sub-diploid peak, suggesting cells were dying by apoptosis.

The SW480 cell line showed no response to treatment with 100 \textmu M indomethacin, however treatment with the higher concentration (200 \textmu M) again led to a significant decrease in the proportion of cells in S and G\textsubscript{2}/M phase; this effect was seen at both 48 and 72 hours. No increase was seen in the proportion of cells in G\textsubscript{0}/G\textsubscript{1}, however a large sub-diploid peak was again seen, indicating that cells might be dying by apoptosis.

Treatment of the colonic cell lines with 5-ASA did not alter the cell cycle distribution; whilst graphs indicate that there may be a decrease in the percentage of HT-29 cells in G\textsubscript{2}/M at the 72 hour time point, this was not found to be statistically significant.
Overall, indomethacin was found to result in a decrease in the percentage of cells in the 
$G_2/M$ and $S$ phases of the cell cycle, suggesting that treatment of these human colonic 
cell lines with indomethacin results in a $G_0/G_1$ phase block. The appearance of a sub-
diploid peak corresponds to the induction of apoptosis following indomethacin treatment. 
5-ASA treatment was not found to have any significant effect on cell cycle distribution 
and the appearance of a sub-diploid peak following 5-ASA treatment was not noticed; 
these result suggested that 5-ASA treatment of cell lines did not lead to the induction of 
apoptosis.
Figure 4.14 DNA content FACS histograms of HCT-15 cells treated with indomethacin or 5-ASA

Cells were treated with 100 or 200 μM indomethacin or 5-ASA at 24 hourly intervals. At 48 hours cells were harvested and fixed, and DNA stained with propidium iodide. DNA content was determined by FACS analysis, and the percentage of cells in the Go/Gi and G2/M phases of the cell cycle determined. Indomethacin treatment decreased the proportion of cells in the S and G2/M (E) phases of the cell cycle and increased the proportion of cells in G0/G1 (D). The novel peak (C) seen following 200μm indomethacin treatment represents cells undergoing apoptosis. 5-ASA treatment did not alter cell cycle distribution.

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Figure 4.15 Effect of 5-ASA and indomethacin treatment on cell cycle distribution of CaCo-2 cells.

Cells were treated with 200 or 100 μM 5-ASA/indomethacin at 24 hourly intervals. 24, 48 and 72 hours following the initial treatment, cells were harvested and cell cycle distribution analysed by flow cytometry as described in materials and methods. Graphs represent ratio of cells in G0/G1 and G2/M as compared to untreated (DMSO only) controls at the time points indicated. Values shown represent the mean value obtained from four analyses; bars indicate SEM. Indomethacin treatment resulted in a dose dependant decrease in the number of cells in G2/M and an increase in the number of cells in G0/G1 (*P<0.05). No effect on cell cycle distribution was seen following 5-ASA treatment.
Figure 4.16 Effect of 5-ASA and indomethacin treatment on cell cycle distribution of HCT-15 cells.

Cells were treated with 200 or 100 μM 5-ASA/indomethacin at 24 hourly intervals. 24, 48 and 72 hours following the initial treatment, cells were harvested and cell cycle distribution analysed by flow cytometry as described in materials and methods. Graphs represent ratio of cells in G0/G1 and G2/M as compared to untreated (DMSO only) controls at the time points indicated. Values shown represent the mean value obtained from four analyses; bars indicate SEM. Indomethacin treatment resulted in a dose dependant decrease in the number of cells in G2/M (*P<0.05). No effect on cell cycle distribution was seen following 5-ASA treatment.
Figure 4.17 Effect of 5-ASA and indomethacin treatment on cell cycle distribution of HT-29 cells.

Cells were treated with 200 or 100 µM 5-ASA/indomethacin at 24 hourly intervals. 24, 48 and 72 hours following the initial treatment, cells were harvested and cell cycle distribution analysed by flow cytometry as described in materials and methods. Graphs represent ratio of cells in $G_0/G_1$ and $G_2/M$ as compared to untreated (DMSO only) controls at the time points indicated. Values shown represent the mean value obtained from four analyses; bars indicate SEM. Indomethacin treatment resulted in a dose dependant decrease in the number of cells in $G_2/M$, accompanied by an increase in the number of cells in $G_0/G_1$ (*P<0.05, **P<0.01). A possible decrease in the number of cells in $G_2/M$ was seen at 72 hours following 5-ASA treatment, however this is not statistically significant.
Figure 4.18 Effect of 5-ASA and indomethacin treatment on cell cycle distribution of SW480 cells.

Cells were treated with 200 or 100 μM 5-ASA/indomethacin at 24 hourly intervals. 24, 48 and 72 hours following the initial treatment, cells were harvested and cell cycle distribution analysed by flow cytometry as described in materials and methods. Graphs represent ratio of cells in G₀/G₁ and G₂/M as compared to untreated (DMSO only) controls at the time points indicated. Values shown represent the mean value obtained from four analyses; bars indicate SEM. 200μM Indomethacin treatment resulted in a decrease the number of cells in G₂/M (*P<0.05, **P<0.01). No effect on cell cycle distribution was seen following 5-ASA treatment.
Table 4.3 Effect of 200 μM 5-ASA or indomethacin treatment on human colonic cell lines (over page)

Cells were treated with 200 μM 5-ASA or indomethacin (Indo) at 24 hourly intervals for 72 hours. Cell number was determined by trypan blue cell counts, and apoptosis was detected by immunohistochemistry using the anti-caspase-3 (active) antibody. In addition, cell viability and apoptosis were measured by flow cytometry following dual staining with PI and annexin-V-FITC. Position of cells within the cell cycle was determined by flow cytometry following fixing of cells and staining of DNA with PI. The cell number, number of viable and apoptotic cells, and proportion of cells in G0/G1 is expressed as a ratio compared to untreated controls. Values shown represent mean values obtained from four experimental repeats. Results were analysed using the paired t-test. Arrows, alongside P value obtained, indicate significant changes in treated groups compared to untreated controls.
### Cell Counts / Immunohistochemistry

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### Flow cytometry - Cell Viability

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### Flow Cytometry – Cell Cycle

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Table 4.3 Effect of 200 μM 5-ASA or indomethacin treatment on human colonic cell lines
4.4 Discussion

The studies presented in this chapter were designed firstly to characterise various human colonic cell lines, and secondly to ascertain the effects of indomethacin and 5-ASA treatment on these cell lines, in particular with regard to cell viability, induction of apoptosis and distribution of cell cycle.

The cell lines of choice, CaCo-2, HCT-15, HT-29 and SW480 are well characterised within existing literature and were chosen for these studies due to their differing Cox-1 and Cox-2 status. Immunohistochemical and PCR analysis of cell lines was carried out in order to detect the presence of p53 protein, and Cox-1 and -2 protein and mRNA. P53 protein was detected in the HCT-15 and SW480 cell lines only. Whilst CaCo-2 is known to express p53 protein, the protein is truncated due to a mutation within the DNA, which results in the formation of a stop codon at position 240, and the protein is non-functional. This truncated p53 protein was not detected by the antibody used in these studies. Analysis of p53 by western blotting would have been a useful technique for detection of the truncated protein.

SW480 is stated in the literature to be p53 negative; positive staining was seen with the p53 antibody, however, the staining appears to be cytoplasmic, which is consistent with a mutated form of p53. The HCT-15 cell line demonstrated positive nuclear staining in a small number of cells; this is likely to be seen in cells that express wild-type p53, as p53 is normally expressed during proliferation. Whilst the literature states that the HCT-15
cell line carries a mutant form of p53, this cannot be confirmed through these results. Further analysis regarding the functionality of p53 could be carried out by immunohistochemical detection of mdm-2, the expression of which is induced by wild-type p53. HT-29 showed no p53 immunoreactivity, which is in agreement with published literature.

RT-PCR analysis of Cox expression in cell lines showed expression of Cox-2 mRNA in HT-29 and, at a higher level, in the CaCo-2 cell line, these findings were confirmed by immunohistochemical detection of Cox-2 which detected low levels of Cox-2 protein in HT-29 cells, and high levels in the CaCo-2 cell line. Cox-2 is believed to be inducible in the HT-29 cell line, and this may be reflected by the fact that only a few cells showed immunoreactivity. In CaCo-2 cells however, Cox-2 immunoreactivity was seen in all cells suggesting that this cell line may carry a mutation resulting in Cox-2 over-expression, indeed Cox-2 is known to be frequently over-expressed in human colonic carcinomas (Eberhart et al, 1994).

The HT-29 and SW480 cell lines both appear to be positive for Cox-1. Existing literature states that SW480 is a Cox negative cell line, the results of this study would challenge that, however no comment can be made as to the functionality of Cox-1. No Cox-1 expression was detected in the CaCo-2 cell line, which is reported to be Cox-1 positive, detection of the protein with the antibody however did give positive results. The absence of Cox-1 mRNA, but apparent presence of protein could be due to various factors; firstly, the CaCo-2 cell line was shown to be positive for Cox-2 protein, and it may be that the
Cox-1 antibody shows some degree of cross-reactivity with the Cox-2 isoform, which would lead to a false positive result. Secondly, Cox-1 may be expressed at very low levels in this cell line and was not detected by RT-PCR.

The HCT-15 cell line expressed very low levels of Cox-1 mRNA but was negative for protein. This could be due to the presence of a mutation within the Cox-1 gene, resulting in absence of translation or the production of a mutated isoform that is not detected by the antibody.

In summary, both the HT-29 and CaCo-2 cell lines were shown to be Cox-2 positive, whilst HCT-15 and SW480 cells were negative. HT-29 and SW480 cells appeared positive for Cox-1, whilst HCT-15 contained neither Cox-1 nor Cox-2 protein. The Cox-1 status of the CaCo-2 cell line remains unclear and further work in order to clarify whether or not this cell line produces Cox-1 transcripts.

Treatment of all cell lines with indomethacin resulted in a dose dependant decrease in cell number, accompanied by an increase in apoptosis as detected by the caspase-3 antibody. The largest reduction in cell number following treatment was seen in the CaCo-2 and SW480 cell lines. Interestingly the CaCo-2 cell line also had the smallest increase in apoptosis. This suggests that that alternative mechanisms may primarily be responsible for the inhibition of cell growth that is seen following treatment in this particular cell line. Other cell lines showed similar sensitivity to indomethacin treatment, indicating that the reduction of cell number and induction of apoptosis is not dependant on p53, Cox-1 or -2
status or tumour grade. Treatment of cells with 200 μM 5-ASA significantly reduced cell number in the HT-29 and SW480 cell lines. No increase in apoptosis was seen following 5-ASA treatment, suggesting the observed reduction in cell number was not due to the induction of apoptosis.

A reduction in cell viability accompanied by the induction of apoptosis following treatment with indomethacin, was also shown following analysis by flow cytometry. Again, this was not shown to be dependant on p53, Cox-1 or -2 expression, or tumour grade. The CaCo-2 and SW480 cells were again shown to be most sensitive to the effects of indomethacin. The SW480 cell line was shown to be particularly sensitive and demonstrated a 25-fold increase in the level of apoptosis following treatment with 200 μM indomethacin for 72 hours. In contrast to results obtained by immunohistochemistry, analysis of apoptosis by flow cytometry showed a high level of induction in the CaCo-2 cell line. These experiments suggest that flow cytometry was more successful in the detection of apoptosis than immunohistochemical techniques; this may be due to accumulation of apoptotic cells during detection by flow cytometry. For immunohistochemical analysis, cells are fixed before detection of apoptosis, resulting in a "snap-shot" picture of apoptosis levels. During detection of apoptosis by flow cytometry however, cells are stained with annexin-V before fixing. This may result in detection of apoptosis over a wider time-frame, leading to apparently higher apoptosis levels following detection by flow cytometry, in comparison to values obtained by immunohistochemical analysis. Furthermore, the cleaved caspase-3 epitope may not be detected during later stages of apoptosis, while the phoshatidylserine flip (detected by
annexin-v) may remain present. This would result in apparently higher levels of apoptosis following detection by flow cytometry, due to the fact that cells can be detected for a longer period of time during the apoptotic process. Staining of cells with annexin-V (for analysis of apoptosis by flow cytometry) is carried out before trypsinisation; original experiments, where cells were stained with annexin-V after trypsinisation resulted in very high levels of necrotic cell death, it is therefore possible that the trypsinisation of the cells during the cytopsin protocol may result in induction of necrotic cell death, leading to break-down of the cells and masking the level of apoptosis.

Whilst indomethacin did reduce cell viability and increase apoptosis in the HCT-15 cell line this was not shown to be statistically significant. This is likely to be due to variations between experiments, which mask a significant result. Whilst each individual experiment demonstrated a decrease in cell viability and an increase in apoptosis following indomethacin treatment, the base line of these values varied in control samples (and therefore in treated samples) between experimental repeats. This could be due to changes within the cell line as passage number increases, experimental error in seeding, or differences in growth medium. To overcome these problems, future experiments for each cell line should be carried out simultaneously, so the same passage number is used to seed each experimental repeat.

Treatment of cell lines with 5-ASA did not alter cell viability or apoptosis levels. Cell counts carried out before immunohistochemical analysis demonstrated a significant decrease in cell number following 5-ASA treatment at the 72 hour time point in HT-29
and SW480 cells; analysis by flow cytometry does not disprove this, as it is the proportion of cells which are viable is being measured, rather than the total cell number.

Overall the studies carried out in this chapter suggest that 5-ASA may decrease cell number in some human colonic cell lines, however this is not occurring through a reduction in cell viability or an increase in apoptosis. A reduction in cellular proliferation may be an important mechanism for chemoprevention by the 5-ASA compounds.

In order to gain further insight into the possible mechanisms for the reduction in cell number and induction of apoptosis in cell lines, studies were carried out to examine cell cycle distribution following indomethacin or 5-ASA treatment. Treatment of all cell lines with indomethacin resulted in cell cycle accumulation in Go/G1, with a concurrent decrease in the proportion of cells the S and G2/M phases. This is in agreement with results published by others (Shiff et al, 1995), this accumulation at the G1 checkpoint seem to occur independently of p53 activation, as these cell lines are known to carry mutant forms of p53. Further studies analysing induction of p21 expression would be useful in determining whether or not p21 could function in a p53 independent manner, and therefore still result in G1 cell cycle accumulation, or if alternative mechanisms are underlying.

The HT-29 cell line showed the greatest G1 phase accumulation following indomethacin treatment. In contrast to this, the SW480 cell line shows a decrease in the number of cells in both the Go/G1 and G2/M phases of the cell cycle. These results suggest that
different pathways may be activated in these two cell lines following indomethacin treatment. The SW480 cell line also showed a large increase in the number of apoptotic cells, it is therefore possible that cells are progressing through the G1 check-point before apoptosis is being triggered. Again, analysis of the p21 protein with regard to its activation may provide further information as to the underlying molecular pathways activated. The presence of sub-diploid peaks, indicating apoptotic cells was seen following indomethacin treatment in both the HCT-15 and SW480 cell lines, whether or not the induction of apoptosis is occurring secondary to cell-cycle inhibition is not clear from these experiments, however this seems unlikely as the cell lines showing the greatest induction in apoptosis (CaCo-2 and SW480) do not both show the greatest inhibition of cell-cycle (HT-29 and SW480). In order to clarify whether or not apoptosis is linked to cell-cycle inhibition, experiments could be carried out on non-proliferating cell lines.

No significant alterations to the cell cycle were seen following 5-ASA treatment suggesting that any chemopreventive effects of this drug act through alternative pathways. A small decrease in the number of cells in G2/M was seen at the 72-hour time point following 5-ASA treatment in the HT-29 cell line, however this is not statistically significant. This may be due to this cell line being particularly sensitive to disruptions in the cell cycle (HT-29 cells were the most sensitive to indomethacin) and therefore an effect is seen with 200 μM 5-ASA in this cell line only, whilst it may take a higher drug concentration to elicit a response in the other cell lines, however the reduction seen may be purely due to variation in data. The observed reduction in G2/M in this cell line is in
contrast to results published by Reinacher-Schick et al (2003), who observed a G2/M phase block following treatment of HT-29 cells with 5-ASA, however, a much greater concentration of 5-ASA was used in these experiments (30 mM).

In conclusion, the studies described in this chapter show that indomethacin treatment of human colonic cell lines results in a reduction in cell number, a decrease in cell viability, induction of apoptosis and accumulation of cells in the G1 phase of the cell cycle. These effects are independent of p53 or Cox expression or tumour grade. Further studies, carried out in primary cell cultures or non-tumorigenic cell lines would be interesting in determining whether the observed induction of apoptosis in response to indomethacin treatment is specific to malignant colonic cells.

There was some evidence for a decrease in cell number following treatment with 5-ASA, however neither immunohistochemical nor flow cytometry analysis showed an induction in apoptosis, furthermore, with the possible exception of the HT-29 cell line, 5-ASA did not alter the cell cycle distribution. These results suggest that whilst 5-ASA may reduce tumour cell number, this is more likely to be due to inhibition of proliferation.
Chapter 5.

The Effect of Indomethacin and 5-ASA Treatment on Bax, Bcl-XL, PPARα, γ and δ Expression in Human Colonic Cell Lines
5.1 Introduction

The mechanisms by which NSAIDS are chemopreventive, and induce apoptosis within colonic tumour cells are presently unclear. The observation that NSAIDS induce apoptosis in Cox-negative cells lines suggests, that whilst the reduction of prostaglandin levels (which are known to increase levels of Bcl-2) through Cox inhibition may be important in the induction of apoptosis and inhibition of tumour formation, Cox inhibition alone is not necessary for the chemopreventive effects of the NSAIDs, and alternative mechanisms exist.

The Bcl-2 family of proteins are well-characterised, important regulators of apoptosis and altered expression of various members of the Bcl-2 family frequently occurs in colonic cancers. There is some evidence to suggest that treatment of colonic cells with NSAIDS results in a reduction in the levels of the anti-apoptotic gene Bcl-XL, therefore increasing the cellular ratio of Bax:Bcl-XL, which in turn favours the induction of apoptosis (Zhang et al, 2000). Whilst there is evidence to suggest 5-ASA compounds are chemopreventive, it is not known if they also induce apoptosis through alterations in the ratio of pro:anti apoptotic members of the Bcl-2 family.

A further, alternative mechanism for chemoprevention by NSAIDS is through the PPAR pathway. Various NSAIDS have been shown to be capable of binding directly to, and activating the PPAR group of nuclear hormone receptors (Lehmann et al, 1997). Three PPAR isoforms known as α, γ and δ have been characterised. Following activation, all three isoforms bind to peroxisome proliferator response elements (PPRE’s) in the promoter region of target genes, and initiate transcription. The target genes of the three isoforms have not been fully determined, however, there is considerable evidence to show that activation of PPARs results in altered cell

Activation of PPARγ is thought to have a chemopreventive effect. Experiments carried out in animal models and human colonic cell lines have shown the induction of apoptosis, reduced tumour cell growth, induction of cellular differentiation and G1 cell cycle arrest (see section 1.7.3) following ligand binding to, and therefore activation of PPARγ. In contrast to the γ isoform, PPARδ is thought to confer a tumorigenic phenotype upon the cell. Although the target genes of PPARδ are as yet unknown, PPARδ mRNA is frequently over expressed in colonic cancer cells (He et al, 1999; Gupta et al 2000). Prostaglandins are known to act as ligands for PPARδ and therefore inhibition of prostaglandin production through treatment with NSAIDs may reduce PPARδ activity. NSAIDs have also been shown to prevent binding of the PPARδ transcription complex to DNA through an unknown mechanism.

The effects of PPARα on tumour formation are less clear. IkBα is thought to be a target gene of PPARα, and it is thought that activation of PPARα may result in the induction of apoptosis through inhibition of the NF-κB pathway. However, in contrast to this, activation of PPARα through ligand binding has been shown to result in the formation of hepatocellular tumours in rodent models (although this effect is not seen in humans).

The fact that the PPAR isoforms could be a target of the NSAIDS puts forward an alternative mechanism for the known chemopreventive effects of this group of drugs. To the best of our knowledge, there have been no studies to date carried out examining the effects of 5-ASA on PPAR expression.
The studies presented in this chapter were designed to examine possible mechanistic effects of indomethacin and 5-ASA treatment on human colonic cell lines, with regards to Bax, Bcl-XL, PPARα, γ and δ expression.
5.2 Does Indomethacin or 5-ASA Treatment Alter Expression of Bax or Bcl-XL?

The effects of indomethacin and 5-ASA treatment on Bax and Bcl-XL expression was analysed in the CaCo-2, HCT-15, HT-29 and SW480 cell lines by PCR. Cells were treated with a 200 µM concentration of indomethacin or 5-ASA, dissolved in DMSO at 24 hourly intervals for 24 or 72 hours. 24 hours after the final treatment point, cells were harvested and lysed, cDNA produced from total mRNA and this cDNA used in a PCR reaction as described in materials and methods (see section 2.2.11 and 2.2.12). Products were analysed by agarose gel electrophoresis and viewed under UV light.

Whilst levels of Bax and Bcl-XL did alter between treatments, no consistent increase or decrease in the expression of either gene was seen following treatment with indomethacin or 5-ASA (figure 5.1-5.4). Alterations in band densities between treatments were generally mirrored in both Bax and Bcl-XL analysis, suggesting that differences may be due to differing cDNA concentrations rather than induction or inhibition of mRNA expression.

To further analyse results obtained by PCR, agarose gels were photographed and subjected to analysis using Scion Image. This program was used to measure band density, which was then normalised to GAPDH in order to account for differences in cDNA concentrations between samples. The level of Bax and Bcl-XL mRNA at the 72 hour time point (expressed as a ratio of control cell level) was calculated (figure 5.5).

Analysis by Scion Image confirmed that neither Bax nor Bcl-XL was consistently up- or down-regulated following treatment with 200 µM indomethacin or 5-ASA. Whilst Bcl-XL does appear to be upregulated in all cell samples at the 24 hour time point (data not shown) this was mirrored
by an increase in Bax expression (except in the SW480 cell line). If this increase in expression of Bax and Bcl-X_\text{L} was occurring in the same cells, it is unlikely alter the susceptibility of the cell to undergo apoptosis.

Comparison of Bax:Bcl-X_\text{L} ratios between cell lines and treatment groups (figure 5.6), showed control HT-29 and SW480 cells to have a Bax:Bcl-X_\text{L} ratio of approximately 0.5. The ratio of Bax:Bcl-X_\text{L} was approximately equal in the HCT-15 cell line, and at the 24 hour time point in the CaCo-2 line, however, at 72 hours CaCo-2 cells showed a decrease in Bcl-X_\text{L} expression, resulting in a Bax:Bcl-X_\text{L} ratio of 1.5 and suggesting a cellular tendency to apoptosis. This result probably reflects the fact that the control CaCo-2 cells had become confluent and rapidly died following this time point. Overall, cell lines showed a higher level of Bcl-X_\text{L} than Bax expression in both control and treated samples.

In summary, treatment of colonic cell lines with 200 \text{\mu M} indomethacin or 5-ASA did not appear to alter expression levels of Bax or Bcl-X_\text{L}, and there was no observed increase in the Bax:Bcl-X_\text{L} ratio (figure 5.6). The increase in apoptosis previously demonstrated in human colonic cell lines following treatment with 200 \text{\mu M} indomethacin (chapter 4) does therefore not appear to be controlled through altered expression of Bax/Bcl-X_\text{L}. Whilst no increase in Bax:Bcl-X_\text{L} mRNA levels was observed, these studies did not give any information regarding the translation of these two genes. Immunohistochemistry would be a useful tool in analysing Bax and Bcl-X_\text{L} protein levels.
Figure 5.1 Expression of Bax and Bcl-XL in control (C), indomethacin (I) and 5-ASA (A) treated CaCo-2 cells.

Cells were treated with 200 μM indomethacin, 5-ASA or an equivalent volume of the diluent DMSO only (controls). 24 or 72 hours following initial treatment, cells were harvested and Bax and Bcl-XL expression analysed by PCR as described in materials and methods. GAPDH was used as a control to monitor mRNA levels. PCR products were separated by electrophoresis in a 3% agarose gel. M indicates 100bp DNA marker and N indicates cDNA-negative controls.
Figure 5.2 Expression of Bax and Bcl-X\textsubscript{L} in control (C), indomethacin (I) and 5-ASA (A) treated HCT-15 cells.

Cells were treated with 200 μM indomethacin, 5-ASA or an equivalent volume of the diluent DMSO only (controls). 24 or 72 hours following initial treatment, cells were harvested plus Bax and Bcl-X\textsubscript{L} expression analysed by PCR as described in materials and methods; GAPDH was used as a control to monitor mRNA levels. PCR products were separated by electrophoresis in a 3% agarose gel. M indicates 100bp DNA marker and N indicates cDNA-negative controls.
Figure 5.3 Expression of Bax and Bcl-X<sub>L</sub> in control (C), indomethacin (I) and 5-ASA (A) treated HT-29 cells.

Cells were treated with 200 μM indomethacin, 5-ASA or an equivalent volume of the diluent DMSO only (controls). 24 or 72 hours following initial treatment, cells were harvested and Bax and Bcl-X<sub>L</sub> expression analysed by PCR as described in materials and methods; GAPDH was used as a control to monitor mRNA levels. PCR products were separated by electrophoresis in a 3% agarose gel. M indicates 100bp DNA marker and N indicates cDNA-negative controls.
Figure 5.4 Expression of Bax and Bcl-XL in control (C), indomethacin (I) and 5-ASA (A) treated SW480 cells.

Cells were treated with 200 μM indomethacin, 5-ASA or an equivalent volume of the diluent DMSO only (controls). 24 or 72 hours following initial treatment, cells were harvested and Bax and Bcl-XL expression analysed by PCR as described in materials and methods; GAPDH was used as a control to monitor mRNA levels. PCR products were separated by electrophoresis in a 3% agarose gel. M indicates 100bp DNA marker and N indicates cDNA-negative controls.
Figure 5.5 Expression of Bax and Bcl-X<sub>L</sub> in human colonic cell lines following indomethacin or 5-ASA treatment.

Cells were treated with 200 μM indomethacin (I) or 5-ASA (A) at 24 hourly intervals for 72 hours. Bax and Bcl-X<sub>L</sub> mRNA was detected by RT-PCR and agarose gel electrophoresis. Expression levels were determined by analysis of band density by Scion Image and normalised to GAPDH. Levels of Bax and Bcl-X<sub>L</sub> mRNA are expressed as a ratio of the expression levels in untreated controls. No consistent changes in Bax or Bcl-X<sub>L</sub> expression were seen.
Figure 5.6 Ratio of Bax:Bcl-X\textsubscript{L} expression in control and treated cell lines.

Cells were treated with 200 \textmu M indomethacin or 5-ASA for 72 hours. Bax and Bcl-X\textsubscript{L} mRNA was detected by PCR and expression levels determined by analysis with Scion Image. Neither Indomethacin nor 5-ASA treatment resulted in an increase in the Bax:Bcl-X\textsubscript{L} ratio.
5.3 Does Indomethacin or 5-ASA Treatment Alter Expression of PPARα, γ or δ?

Experiments were designed to analyse the effects of indomethacin or 5-ASA treatment on PPARα, γ and δ expression in human colonic cell lines. Cells were treated with a 200 μM concentration of indomethacin or 5-ASA at 24 hourly intervals for 24 or 72 hours. 24 hours after the final dose, cells were harvested, lysed and cDNA produced. PPARα, γ and δ expression was measured by PCR (figures 5.7–5.10) and the resulting bands analysed by Scion Image as described for the Bax/Bcl-XL studies (section 5.2).

PPARδ expression was lowest in the HT-29 cell line and appeared to be induced slightly by indomethacin or 5-ASA treatment (data not shown). All cell lines with the exception of CaCo-2 appeared to show some induction of PPARδ expression following indomethacin treatment at the 72 hour time point, however these observations should be treated with caution as despite numerous experimental repeats, amplification of PPARδ appeared inconsistent and in some samples was completely undetectable; it is therefore not possible to draw any conclusions regarding the effects of indomethacin or 5-ASA treatment on PPARδ expression.

Amplification of PPARα and γ gave more consistent results. The levels of PPARα mRNA in control samples was similar for all cell lines, and whilst treatment with 200 μM indomethacin had no effect at 24 hours, a significant increase in PPARα transcription was seen in all four cell lines following indomethacin treatment at the 72 hour time point (P=0.012) (figure 5.11). At 72 hours, PPARα expression was approximately 1.4 fold higher in indomethacin treated samples, in comparison to controls.
Treatment of cell lines with 5-ASA did not consistently increase PPARα levels. The CaCo-2 and HCT-15 cell lines showed an increase in PPARα expression following 5-ASA treatment at the 72 hour time point; however in contrast to this, analysis of the HT-29 and SW480 cell lines showed reduced PPARα expression following 5-ASA treatment.

Unlike PPARα, levels of the PPARγ isoform in control samples varied widely between cell lines. HCT-15 and HT-29 had low levels of PPARγ mRNA; in comparison, levels were approximately 3 fold higher in SW480 cells and 10 fold higher in the CaCo-2 cell line. Treatment of all four cell lines with 200 µM indomethacin resulted in a significant increase in PPARγ transcription (P=0.015) (figure 5.12), the greatest increase was seen in the HCT-15 cell line, which showed very low levels of transcription in untreated cells.

Treatment of cells with 200 µM 5-ASA also resulted in a significant increase in PPARγ expression (P=0.046). HT-29 cells demonstrated the greatest increase in PPARγ expression following 5-ASA treatment, with an approximate 4-fold increase in mRNA levels in comparison to the control. Whilst treatment of CaCo-2, HCT-15 and SW480 cell lines with 5-ASA also increased PPARγ expression, the effect was less than that which was seen following indomethacin treatment.

Overall, these results demonstrate that 200 µM indomethacin treatment of the human colonic cell lines CaCo-2, HCT-15, HT-29 and SW480 results in the up-regulation of PPARα and γ expression; furthermore, treatment of the same cell lines with 200 µM 5-ASA resulted in increased PPARγ expression at the 72-hour time point.
Cells were dosed daily with 200 μM indomethacin, 5-ASA or an equivalent volume of the diluent DMSO only (control). 24 or 72 hours following initial treatment cells were harvested and PPARα, γ and δ expression analysed by PCR as described in materials and methods. PCR products were separated by electrophoresis in a 3% agarose gel. M indicates 100bp DNA marker and N indicates cDNA-negative controls.
Cells were dosed daily with 200 μM indomethacin, 5-ASA or an equivalent volume of the diluent DMSO only (control). At 24 or 72 hours following initial treatment cells were harvested and PPARα, γ and δ expression analysed by PCR as described in materials and methods. PCR products were separated by electrophoresis in a 3% agarose gel. M indicates 100bp DNA marker and N indicates cDNA negative controls. Results indicate up-regulation of PPARα and γ in both indomethacin and 5-ASA treated cells at the 72 hour time-point.
Figure 5.9 Expression of PPARα, γ and δ in control (C), indomethacin (I) and 5-ASA (A) treated HT-29 cells.

Cells were dosed daily with 200 μM indomethacin, 5-ASA or an equivalent volume of the diluent DMSO only (control). 24 or 72 hours following initial treatment cells were harvested and PPARα, γ and δ expression analysed by PCR as described in materials and methods. PCR products were separated by electrophoreses in a 3% agarose gel. M indicates 100bp DNA marker and N indicates cDNA-negative controls. Results at the 72-hour time point indicate upregulation of PPARα and γ following indomethacin treatment, and upregulation of PPARγ following 5-ASA treatment.
Figure 5.10 Expression of PPARα, γ and δ in control (C), indomethacin (I) and 5-ASA (A) treated SW480 cells.

Cells were dosed daily with 200 μM indomethacin, 5-ASA or an equivalent volume of the diluent DMSO only (control). 24 or 72 hours following initial treatment cells were harvested and PPARα, γ and δ analysed by PCR as described in materials and methods. PCR products were separated by electrophoresis in a 3% agarose gel. M indicates 100bp DNA marker and N indicates cDNA-negative controls. Results indicate up-regulation of PPARα and γ following indomethacin or 5-ASA treatment at the 72 hour time point.
Figure 5.11 Expression of PPARα in human colonic cell lines following indomethacin or 5-ASA treatment.

Cells were treated with 200 μM indomethacin (I) or 5-ASA (A) at 24 hourly intervals for 72 hours. PPARα expression was detected by RT-PCR and agarose gel electrophoresis. Band densities were analysed by Scion Image and expression levels normalised to GAPDH. PPARα expression is shown as a ratio of expression levels in untreated controls. PPARα expression was significantly increased in cell lines following indomethacin treatment (P=0.012).
Figure 5.12 Expression of PPARγ in human colonic cell lines following indomethacin or 5-ASA treatment.

Cells were treated with 200 µM indomethacin (I) or 5-ASA (A) at 24 hourly intervals for 72 hours. PPARγ expression was detected by RT-PCR and agarose gel electrophoresis. Band densities were analysed by Scion Image and expression levels normalised to GAPDH. PPARγ expression is shown as a ratio of expression levels in untreated controls. PPARγ expression was significantly increased in cell lines following 5-ASA (P=0.046) or indomethacin (P=0.015) treatment.
5.4 Discussion

The studies described in this chapter were designed to examine possible Cox-2 independent pathways for the induction of apoptosis that is seen following treatment of human colonic cell lines with indomethacin. Experiments were designed to analyse changes in Bax, Bcl-X\textsubscript{L}, PPAR\(\alpha\), \(\gamma\) and \(\delta\) expression following treatment with 200 \(\mu\)M indomethacin, the effects of 5-ASA treatment (which has been reported by others to be chemopreventive), on gene expression was also examined.

Zhang et al (2000), reported NSAID inhibition of expression of the anti-apoptotic protein Bcl-X\textsubscript{L}. This results in an increase in the Bax:Bcl-X\textsubscript{L} ratio within the cell, with a subsequent increase in cellular apoptosis. Alterations in the levels of Bax and Bcl-X\textsubscript{L} therefore present a possible Cox-independent pathway for the induction of apoptosis. The data presented in this chapter however, indicated that neither indomethacin nor 5-ASA treatment resulted in an increase in the Bax:Bcl-X\textsubscript{L} ratio in any of the cell lines tested. The increase in Bax:Bcl-X\textsubscript{L} ratio seen in the HCT-15 cell line following 5-ASA treatment at 72 hours (figure 5.5) is likely to be due to the fact that this was a rapidly growing cell line, and the sample had become confluent by the final time point.

The results presented in this chapter represent data obtained from one experiment only and to verify these results, it would be necessary to carry out repeated experiments. Furthermore, Bax is prone to mutations in colonic cell lines of a mismatch repair deficient phenotype (HCT-15) due to an unstable G\textsubscript{8} region found at nucleotides 114 to 121; it would therefore be of interest to fully characterise the genotype and phenotype of each cell line in order to see if Bax phenotype correlates with induction of apoptosis by
indomethacin; however as no increase in Bax:Bcl-X \textsubscript{L} was seen, this is unlikely to be an important factor in chemoprevention by indomethacin.

Existing literature suggests that Bcl-X \textsubscript{L} may play an important role in tumorigensis as it is reported to be over-expressed in a large proportion of colorectal carcinomas (Maurer et al, 1998). Results described in this chapter however, suggest that the chemopreventive, effects of indomethacin are not due to its ability to decrease Bcl-X \textsubscript{L} expression, nor was there any increase seen in Bax expression. Overall, it appears that whilst indomethacin induces apoptosis in human colonic cell lines, this is not mediated through the Bax:Bcl-X \textsubscript{L} pathway. Similarly, 5-ASA had no effect on Bax or Bcl-X \textsubscript{L} expression, however, 5-ASA was not shown to induce apoptosis in colonic cell lines (chapter 4) and it is likely that the chemopreventive effects of 5-ASA, which have been reported by others, may not be due to induction of the apoptotic pathway.

The data presented in section 5.3 of this chapter examined the effects of indomethacin and 5-ASA treatment on PPAR \textsubscript{α}, \textsubscript{γ} and \textsubscript{δ} expression. Indomethacin was shown to significantly increase both PPAR\textsubscript{α} and \textsubscript{γ} expression in all cell lines. Indomethacin has previously been shown to activate PPAR\textsubscript{γ} (Lehmann et al, 1997), and is thought to be capable of directly binding to, and therefore acting as a ligand to PPAR\textsubscript{γ}. Activation of PPAR\textsubscript{γ} has been shown to reduce tumour cell growth in mice, result in G\textsubscript{1} cell cycle arrest, and induce apoptosis in colonic cell lines. A number of PPAR\textsubscript{γ} target genes have been identified that are associated with cellular proliferation and differentiation (section 1.7.3) and these go some way to explaining the possible chemopreventive effects of indomethacin treatment. Whether or not PPAR\textsubscript{γ} expression is linked to the induction of apoptosis, and if so the pathways through which this occurs, are as yet unknown and
further work is needed to characterise the target genes of PPARγ. Treatment of cell lines with 5-ASA was also shown to significantly increase PPARγ expression at the 72-hour time point; This may in part explain why 5-ASA has been found to be chemopreventive by others in epidemiological or animal model studies, but failed to induce apoptosis in cultured colonic cell lines. Thus the chemopreventive mechanism of 5-ASA may act via different pathways that do not involve induction of apoptosis.

Indomethacin treatment resulted in increased PPARα expression at the 72-hour time point. Again, the target genes of PPARα are not fully known, however, the α isoform is thought to reduce NF-κB activity through induction of IκB expression, as well as by direct binding to the p65 NF-κB subunit. Overall, these actions prevent NF-κB translocation to the nucleus where it results in the induction of target genes associated with cell survival and proliferation. Therefore, the activation of PPARα by indomethacin may represent a possible pathway for the decrease in cell survival and the induction of apoptosis, which is seen following treatment. The effects of PPARα activation on cells however is still unclear, and conflicting reports regarding chemoprevention verses tumorigenesis exist (section 1.7.2), therefore, whilst this data suggests that indomethacin results in the upregulation of PPARα expression the consequences of this on the cell are not clear and further work to characterise PPARα target genes is needed.

Treatment of cell lines with 5-ASA resulted in an increase in PPARα expression in the CaCo-2 and HCT-15 cell lines, but a decrease in the HT-29 and SW480 cell lines. These changes were not as great as those seen following indomethacin treatment and could be due to experimental variance, experiments should be repeated in order to clarify results.
Unlike PPARα, which demonstrated similar expression levels in all cell lines, the levels of PPARγ in control samples varied widely between cell lines. CaCo-2 showed the highest levels of PPARγ and this may reflect the fact that this particular cell line was well differentiated and the slowest growing of all cell lines used. HT-29 cells, however, which were well differentiated, but a fairly rapid growing cell line, had low levels of PPARγ expression.

The detection of PPARδ mRNA gave inconsistent results. This may be due to the design of the primers, and in order to gain further information regarding PPARδ expression further experiments would have to be carried out. Work carried out by others has shown PPARδ to be a target of various NSAIDs. It is thought that certain NSAIDs are able to directly bind to and inhibit the DNA binding activity of PPARδ. Whilst the target genes of PPARδ are unknown, PPARδ mRNA has been shown to be frequently over expressed in colonic cancer cells (He et al 1999; Gupta et al 2000) therefore suggesting that the target genes may contribute to a malignant phenotype. Furthermore PPARδ is known to be a down-stream target of the β-catenin/TCF-4 transcription complex. APC is frequently mutated in colonic tumours, and in the absence of this protein β-catenin is free to translocate to the nucleus, here it forms a transcription complex with TCF-4 and this complex induces the expression of various genes that promote cell survival and differentiation. NSAIDs may therefore play a chemopreventive role by compensating for the mutant APC; whilst β-catenin will still form a transcription complex with TCF-4, and PPARδ expression will be induced by this, the presence of the NSAID (which is known to interact with PPARδ) prevents PPARδ binding to promotor regions in DNA and inducing possible further tumorigenic gene expression. Interestingly, the HT-29 cell line, which had far lower levels of PPARδ expression in comparison to other cell lines, is the
only cell line that does not have allelic loss of APC (Rowan et al, 2000). This may explain the moderately well differentiated phenotype of this cell line, despite low observed PPARγ expression.

Due to limited time, the results presented in this chapter were obtained from one experiment only. However, the observed increases in PPARγ and PPARα and γ expression following 5-ASA and indomethacin treatment respectively were seen in all four cell lines used in these studies. In order to confirm these initial observations repeated analysis should be carried out. The use of real time PCR would allow more accurate quantification of PPAR expression levels in normal and treated cell samples.

In conclusion, the induction of apoptosis in colonic cell lines following treatment with the NSAID indomethacin does not appear to be mediated through the Bax:Bcl-XL pathway, and it would appear that alternative mechanisms are underlying. PPARα and γ expression is increased in cell lines following indomethacin treatment; whilst the target genes of these nuclear receptors are not known, it is thought they confer a chemopreventive effect upon the cell, due to induction of differentiation and alterations to cell proliferation, whether or not induction of PPAR expression is linked to the induction of apoptosis is not known. 5-ASA was also shown to induce PPARγ expression, and this may present an alternative pathway for the reported chemopreventive effects of this compound.
Chapter 6.

Concluding Remarks and Areas for Further Study.
Cell turnover within the colon is high and this may, in part, explain why the colon appears prone to the development of tumours; by the age of 70, 50% of the western population will have some form of colonic tumour. UC is a chronic relapsing inflammatory disorder, the aetiology of which is unknown. The presence of longstanding UC disease has been shown in epidemiological studies to predispose to the development of colorectal cancer (Ekbom et al, 1990). Patients with UC are frequently treated with 5-ASA, a compound that is similar in structure to the NSAID aspirin. Aspirin, along with various other NSAIDs has been shown in various epidemiological studies to be chemopreventive (Waddell and Loughry 1983; Kune et al, 1988; Thun et al 1993) and this chemopreventive ability is thought to be due to the induction of apoptosis (Shiff et al 1995; Hong et al 1998). Limited studies have demonstrated various possible chemoprevetive properties of 5-ASA (Bus et al, 1999; MacGregor et al, 2000) however there are limited studies on this, and the mechanisms by which NSAID and 5-ASA may prevent colonic neoplasia remain unknown.

The studies presented in this thesis were designed firstly to analyse alterations in cell survival and proliferation in the development of various pathological disorders within the colon, and secondly, to examine the effect of 5-ASA and indomethacin treatment on colonic cell turnover, and changes in the expression of genes that may be associated with cell survival, apoptosis or differentiation.

Both apoptosis and proliferation levels were found to be frequently altered in the development of UC, this increase in cell turnover may explain a predisposition to neoplasia. The increase seen in apoptosis is likely to be the cause of the breakdown of the epithelial barrier which occurs in severe UC. Treatment of patients with 5-ASA was shown to significantly reduce apoptosis in colonic epithelial cells. These results suggested that 5-ASA may actually limit
apoptosis in colonic epithelium, and therefore induction of apoptosis (as is seen with NSAIDs) is unlikely to be the underlying mechanism for possible chemopreventive properties. Studies carried out on various colonic adenocarcinoma cell lines failed to show any alterations in apoptosis levels following 5-ASA treatment. This discrepancy may be due to various experimental differences between the in vivo and in vitro systems. Firstly, the concentrations of 5-ASA may differ between in vivo and in vitro studies, and secondly 5-ASA may undergo differing metabolism in the body in comparison to in cell culture. 5-ASA is thought to undergo acetylation once taken up by colonic epithelial cells (Palumbo et al, 1995; Ireland et al, 1990), however within inflamed tissue (such as UC) the presence of reactive oxidants is thought to alter this metabolic pathway, resulting in the formation of 2, 5-dihydroxybenzoic acid (5-HSA) (Ireland et al 1987). It may be that the acetyl metabolite is crucial for chemoprevention and the induction of apoptosis whilst 5-HSA does not possess these properties.

Further insight into the metabolites produced, and their action on cell turnover could be gained through the comparison of colonic tissue taken from normal and inflamed regions of UC suffers, 5-ASA and it's metabolites could be detected by HPLC and apoptosis and proliferation detected by immunohistochemistry, in addition, oxidative stress could be induced in cell lines by treatment with H$_2$O$_2$, and the effects of 5-ASA on control and stressed cells compared. It is possible that the concentrations of 5-ASA used within these studies were too low to induce apoptosis; further studies with increased concentrations of 5-ASA could be carried out for clarification.

NSAIDS such as indomethacin have been shown by others to be chemopreventive within the colon and it is believed that this is due to their ability to induce apoptosis (see chapter 1,
Data shown in chapter 4 showed a reduction in cell number, reduced cell viability, an increase in apoptosis, and accumulation of cells in the G₀/G₁ phase of the cell cycle following treatment of colonic cells lines with indomethacin. The sensitivity of cells to these effects did not correlate with differentiation status or Cox expression.

Immunohistochemical studies suggested a decrease in proliferation following 5-ASA treatment in the HT-29 and SW480 cell lines (chapter 4). However no significant changes in apoptosis levels or cell cycle distribution were seen. These results suggest that whilst 5-ASA may reduce cell number in some human colonic adenocarcinoma cells, this reduction is not due to the inhibition of cell cycle or the induction of apoptosis, and that the chemopreventive mechanisms of indomethacin and 5-ASA may differ.

The induction of apoptosis seen following indomethacin treatment does not appear to be regulated through changes in Bax or Bcl-X₅ expression (chapter 5). Bax over-expression has been shown to be present in colonic adenocarcinomas, (chapter 2), this may result in tumour cells being more sensitive to the apoptosis inducing effects of the NSAIDs, studies on Bax positive and negative cell lines would provide further information regarding the effect of Bax expression on sensitivity to the induction of apoptosis.

Data in chapter 5 showed for the first time that 5-ASA treatment of colonic cells resulted in increased PPARγ expression. PPARγ has been shown to induce transcription of NGAL, which is involved in the negative control of the inflammatory response (Kjeldsen et al, 2000), and is thought to inhibit the expression of various cytokines (Jiang et al, 1998). Activation of PPARγ by 5-ASA may therefore play a role in reducing inflammation in UC.
Activation of PPARγ is also thought to be involved in the promotion of cell differentiation and inhibition of cell proliferation, however despite the fact that 5-ASA was shown to increase PPARγ expression, treatment of UC patients with 5-ASA appeared to lead to an increase in epithelial proliferation (although this was not statistically significant (chapter 3)). The differences in results may be due to the malignant genotypes of the cultured cells. CaCo-2 and both CaCo-2 and Ht-29 cells showed a (non-significant) increase in cell number and cell viability following 5-ASA treatment in immunohistochemical and flow cytometric studies respectively, suggesting that treatment may have differing effects in normal, in comparison to malignant cells.

RegIA is a gene involved in cellular proliferation, and has been shown to be over-expressed in a large number of colorectal tumours (see chapter 1; 1,7.3). This gene may be over-expressed in the cell lines examined and therefore inhibition through PPARγ activation may drastically reduce proliferation, whilst in normal or UC epithelium, RegIA expression may be minimal and therefore effects of 5-ASA on proliferation differ. 5-ASA was seen to reduce cell number in the HT-29 and SW480 cell lines only (chapter 4), it would be interesting to examine expression of RegIA in cell lines, to see if inhibition of proliferation by 5-ASA may correlate to cell genotype. Furthermore, expression of PPARγ target genes could be analysed by RT-PCR within cell lines, and by in situ hybridisation, or cell isolation, through laser-capture, followed by RNA analysis, in biopsy tissue. An increase in the level of epithelial cell proliferation may play an important role in the development of colorectal cancer (chapter 3), whilst cell number and viability following 5-ASA treatment was analysed, these studies could have been extended to include detection of cell proliferation by immunohistochemistry using the Ki-67 antibody.
Whilst PPARα and γ expression were both increased following indomethacin treatment it is unlikely that this is linked to the induction of apoptosis as similar increases in expression were seen following 5-ASA treatment, but no increase in apoptosis with treatment was observed.

Whilst indomethacin and other NSAIDs are likely to be chemopreventive due to induction of apoptosis, they are not suitable for this purpose within the general population due to problems with associated gastric and renal toxicity. Cox-2 specific NSAIDs however, are believed to be safe for long-term use, and show minimal side-effects. The effects of these Cox-2 specific inhibitors on cell proliferation, apoptosis, and cell cycle and comparison of these with the effects of non-specific NSAIDs would be useful in determining the potential chemopreventive effects of COX-2 inhibitors.

In conclusion, deregulation of the normal cell growth and death control plays a vital role in the development of colonic neoplasia, the increase in cell turnover that is seen in UC increases mutation rates and therefore predisposes to the development of colonic cancer. NSAIDs are capable of reducing cell viability and increasing apoptosis within malignant colonic cells, the mechanisms underlying this increase in apoptosis are unclear, however it does not appear to be mediated through increase in Bax, or decrease in Bcl-XL expression. Indomethacin and both 5-ASA and indomethacin have been shown to increase PPARα and PPARγ expression respectively; whilst this may play a role in chemoprevention due to promotion of cell differentiation or via other pathways, an increase in PPARα or γ expression alone does not account for induction of apoptosis, as this was not seen following 5-ASA treatment. The effects of both 5-ASA and NSAIDs are likely to differ in normal to malignant cells, due to both the surrounding environment, and the genetic differences between the two
cell types. Both 5-ASA and Cox-2 specific NSAIDs are believed to be relatively safe for long-term use and could be invaluable in the prevention of colorectal cancer, however more work is needed to unravel the mechanisms by which both the NSAIDs and 5-ASA exert their effects.

The aims of these studies were:

1. To analyse cell turnover in normal, neoplastic and inflamed colonic mucosa, and to examine how changes in cell turnover correlate with Bcl-2 and Bax expression.

Studies presented in chapter 3 show that cell turnover becomes deregulated in colonic neoplasia; overall cell turnover is increased, with high rates of proliferation and increased apoptosis seen to that observed in normal colonic mucosa. Furthermore proliferating cells are not confined to crypt bases as is seen in normal tissue. These observations suggest that deregulation in the control of cell turnover and the proliferation of inappropriate cells contributes to malignant phenotype. The increase in apoptosis seen in colonic neoplasia corresponded to an increase in Bax, and decrease in Bcl-2 expression. Increased levels of apoptosis were seen in the lamina propria of inflamed tissues, this is likely to be due to an increase in inflammatory cell turnover.

2. To analyse and compare cell turnover in quiescent and severe UC in order to gain further insight into the mechanisms that underlie the chronic inflammatory state, and breakdown of colonic mucosa, which are associated with this disorder.
Apoptosis was found to be significantly increased within the lamina propria of severe, compared to quiescent UC; proliferation of both epithelial cells, and cells within the lamina propria was higher in severe UC than in quiescent disease. In severe disease, proliferation levels within the lamina propria appeared much higher than apoptotic rates, and this may contribute to increased inflammatory cell numbers, and contribute to the chronic inflammatory state. The overall increase in cell turnover that is observed in severe UC will pre-dispose to the accumulation of genetic mutations, and this suggests a possible reason for the observed increased risk of colorectal cancer in UC sufferers.

3. To examine the effects of 5-ASA treatment on cell turnover in active UC, in order to shed light on the mechanisms by which this compound may help to induce remission of active disease.

5-ASA treatment was shown to significantly reduce epithelial apoptosis in the colonic mucosa of UC sufferers. The reduction in apoptosis may aid the reconstitution of the epithelial cell barrier, and this may represent a pathway by which 5-ASA acts to bring about remission in active UC.

4. To compare the chemopreventive potential of 5-ASA with the NSAID indomethacin in malignant human colonic cells, in particular with regards to effects on cell viability, apoptosis and cell-cycle distribution

Indomethacin was seen to reduce cell viability and induce apoptosis in a dose-dependant manner. These effects were found to be independent of the Cox or p53 status of the cell lines. Indomethacin treatment resulted in accumulation of cells in the $G_0/G_1$ stage of the cell cycle.
5-ASA was not seen to induce apoptosis in the colonic cell lines used in these studies. Further studies would need to be carried out with increased concentrations of 5-ASA in order to determine whether or not 5-ASA is capable of reducing cell viability and inducing apoptosis in human colonic cells; however these studies suggest that 5-ASA is not capable of reducing cell growth or inducing apoptosis in colonic cell lines at the concentrations at which the NSAID indomethacin is effective.

5. To explore the mechanisms by which chemoprevention by NSAIDs and 5-ASA may occur; in particular with regards to alterations to Bax, Bcl-XL and PPAR expression.

Treatment of human colonic cells with indomethacin or 5-ASA was not found to significantly alter the Bax:Bcl-XL ratio. Indomethacin was found to induce apoptosis in these cell lines, however these results suggest that this is not regulated by the Bax:Bcl-XL pathway. Indomethacin and both 5-ASA and indomethacin were found to up-regulate PPARα and PPARγ expression respectively. This may represent an alternative pathway for the observed and reported chemopreventive properties of NSAIDs and 5-ASA. Further work is needed to characterise the downstream effects of PPARα and γ activation, however, to the best of our knowledge this is the first report of up-regulation of PPARα and γ by 5-ASA and this presents a possible new pathway through which chemoprevention by 5-ASA compounds may occur.
References.


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Publications Relating to this Thesis
