INVESTIGATION INTO THE MECHANISMS OF COLON TUMOUR PROMOTION BY FP-12 AND TNFα AND ITS MODULATION BY CHEMOPREVENTIVE AGENTS

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by
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ABSTRACT

Investigation into the mechanisms of colon tumour promotion by FP-12 and TNFα and its modulation by chemopreventive agents

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The mechanisms whereby two endogenous agents, Fecapentaene-12 (FP-12) and Tumour necrosis factor alpha (TNFα) act as colon tumour promoters was investigated, in human colon epithelial cells (HCEC), a normal colon epithelial cell line. Cyclooxygenase-2 (COX-2), an inducible form of prostaglandin H synthase (PGHS), is induced selectively at the transcriptional level in human colon tumours compared to normal colon epithelium. Exposure to both FP-12 (1-40μM) and TNFα (0.1-10ng/ml), for 2 hours caused a dose dependent, 2 to 6-fold increase in COX-2 mRNA compared to unstimulated cells, when measured by reverse transcription polymerase chain reaction (RT-PCR).

Since inactivation of COX-2 expression has been shown to inhibit colon carcinogenesis, the ability of the chemopreventive agents, sodium salicylate, curcumin, caffeic acid phenethyl ester (CAPE) and resveratrol to inhibit COX-2 mRNA expression was determined. Only curcumin pretreatment, prior to FP-12 or TNFα caused a significant, dose dependent 20-90% inhibition of COX-2 mRNA expression.

The transcription factor nuclear factor kappa B (NF-κB) has been shown to be an important regulator of COX-2 gene expression, and curcumin has been shown to inhibit NF-κB activation. TNFα (1 or 10ng/ml) and FP-12 (20μM) increased the DNA binding of NF-κB to its consensus sequence in gel shift assays by 4 to 5- and 4-fold respectively (relative to unstimulated cells). This was reduced by 50 to 90% in cells pretreated with curcumin prior to TNFα or FP-12.

The ability of curcumin to induce cell death as a possible mechanism for its chemopreventive effects was assessed by the MTT assay, Tryphan blue, sub-G1 analysis, an Annexin method and by DNA laddering gels. Curcumin pretreatment, prior to FP-12 or TNFα appeared to increase cell death, compared to treatment with either agent alone, but no evidence for an apoptotic effect was observed.

Since COX-2 induction by colon tumour promoters may have a pivotal role in colon carcinogenesis, part of the chemopreventive activity of curcumin could be mediated in part through down-regulation of COX-2 gene expression via inhibition of NF-κB activation.
This thesis is dedicated to my mum and dad,
With much love and thanks for everything
Acknowledgements

I would like to thank my supervisor, Dr Simon Plummer for his guidance and enthusiasm throughout my work. I am also greatly thankful to Professor Andy Gesher and Dr Maggie Manson for both encouragement and financial support especially towards the end of my project. I would also like to thank Dr Rebecca Munks for help with the transfection studies, and Roger Snowden for help with the operation of the flow cytometer. I would also like to thank Zara Doddridge, Helen Ball and Roz Hanway, amongst others, who have been very supportive in times of desperation, and whose friendship I greatly value. Finally I would like to thank Dr Steve Faux for his support and sticking with me even when it seemed that this thesis would never be finished.
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>3AB</td>
<td>3-aminobenzamide</td>
<td></td>
</tr>
<tr>
<td>ACF</td>
<td>aberrant crypt foci</td>
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<td>AMPS</td>
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<td>azoxymethane</td>
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<td>activator protein-1</td>
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</tr>
<tr>
<td>AP-2</td>
<td>activator protein-2</td>
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<td>APC</td>
<td>adenomatous polyposis coli</td>
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<td>APHS</td>
<td>o-acetoxyphenylhept-2-ynyl sulfide</td>
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<td>ATP</td>
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<tr>
<td>bp</td>
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</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CAPE</td>
<td>caffeic acid phenethyl ester</td>
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<tr>
<td>CHUK</td>
<td>conserved helix loop ubiquitous kinase</td>
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<tr>
<td>CRE</td>
<td>cyclic AMP response element</td>
<td></td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element DNA binding protein</td>
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<tr>
<td>DAG</td>
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</tr>
<tr>
<td>DCC</td>
<td>deleted in colorectal carcinoma</td>
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<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz(a)anthracene</td>
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<td>DMEM</td>
<td>Dulbecco's Minimal Essential medium</td>
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<td>DMSO</td>
<td>dimethylsulphoxide</td>
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</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>DNTPs</td>
<td>deoxynucleotide triphosphates</td>
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</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
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<tr>
<td>ds</td>
<td>double stranded</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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</tr>
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<td>Abbreviation</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol-bis (β-aminoethyl ether) N,N,N’ N'-tetraacetic acid</td>
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<td>ELAM1</td>
<td>endothelial leukocyte adhesion molecule 1</td>
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<tr>
<td>EMSA</td>
<td>electrophoretic gel mobility shift assay</td>
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<tr>
<td>FADD</td>
<td>Fas associated protein death domain</td>
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<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
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<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>FP-12</td>
<td>fecapentaene-12</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate-dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
<td></td>
</tr>
<tr>
<td>HCEC</td>
<td>human colon epithelial cells</td>
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<tr>
<td>HIV-1</td>
<td>human T-cell leukemia virus type 1</td>
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<td>HNPCC</td>
<td>hereditary non-polyposis colorectal cancer</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>ICAM 1</td>
<td>intracellular adhesion molecule 1</td>
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<td>IκB kinase</td>
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<td>IL-6</td>
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<td>JNK</td>
<td>cJun N-terminal kinase/stress activated protein kinase</td>
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<td>LPS</td>
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<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>mutated in colon cancer</td>
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<td>extracellular regulated kinase kinase kinase 1</td>
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<td>maloney murine leukaemia virus-reverse transcriptase</td>
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<td>mRNA</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazd-2-yl)-2,5-diphenyl tetrazolium bromide</td>
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<tr>
<td>NaAc</td>
<td>sodium acetate</td>
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<td>NaSal</td>
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NAC  N-acetyl-L-cysteine
NF-κB  nuclear factor kappa B
NIK  NF-κB inducing kinase
NSAID  non-steroidal antiinflammatory drug
OD  optical density
ODC  ornithine decarboxylase
8-OHdG  8-hydroxydeoxyguanosine
PBS  phosphate buffered saline
PGE2  prostaglandin E2
PGG2  prostaglandin G2
PGH2  prostaglandin H2
PGHS  prostaglandin H synthase
PGI2  prostaglandin I2
PI  propidium iodide
PKA  protein kinase A
PKC  protein kinase C
PLA2  phospholipase A2
PLC  phospholipase C
PMSF  phenylmethylsulphonyl fluoride
PS  phosphatidylserine
RNA  ribonucleic acid
RNase  ribonuclease
ROS  reactive oxygen species
rpm  revolutions per minute
RSV  Rous sarcoma virus
RT-PCR  reverse transcription polymerase chain reaction
S.D.  standard deviation
SDS  sodium dodecyl sulphate
SDS-PAGE  sodium dodecyl sulphate polyacrylamide gel
S.E.  standard error
TBE  tris- borate EDTA
TBS  tris buffered saline
TBST20  tris buffered saline Tween 20
<table>
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<td>TE</td>
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<tr>
<td>TEMED</td>
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<td>TNFR</td>
<td>tumour necrosis factor receptor</td>
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<td>TPA</td>
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<td>UV</td>
<td>ultraviolet</td>
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<td>VCAM 1</td>
<td>vascular cell adhesion molecule 1</td>
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CHAPTER 1: GENERAL INTRODUCTION
This introduction describes what is known about the mechanisms underlying the development of colon cancer. The model endogenous agents fecapentaene-12 (FP-12) and tumour necrosis factor α (TNFα) are introduced, and the enzyme system which is the focus of this work is described, that of the inducible form of the enzyme prostaglandin H synthase (PGHS). The introduction also outlines ways in which tumour promoter exposure leading to increased expression of early response genes such as cyclooxygenase-2 (COX-2), can be inhibited by conventional non-steroidal antiinflammatory drugs (NSAIDs) and natural dietary constituents, particularly the chemopreventive agents, curcumin, caffeic acid phenethyl ester (CAPE) and resveratrol. These agents have been reported to have chemopreventive efficacy in animal models of colon carcinogenesis. The introduction also describes the signalling pathways that could be important in the regulation of COX-2 expression, particularly the role of the transcription factor nuclear factor kappa B (NF-κB). The role of tumour promoters and chemopreventive agents in the balance between apoptosis and cell proliferation is discussed.

1.1: Colorectal cancer as a multistage process

Colorectal cancer is the second most common cancer in the European Community, with an estimated 135 000 new cases each year (Jensen, 1990). High incidence rates of colorectal cancer are also found in North America and Australasia, intermediate rates in Eastern Europe with the lowest rates found in sub-Saharan Africa (Boyle et al, 1985, Langman and Boyle, 1998). The incidence of colorectal cancer throughout the UK is about 30 000 cases each year resulting in about 17 000 deaths per annum (Tomlinson et al, 1997). Although a small percentage of cases of colorectal cancer occur in individuals with predisposing conditions, such as extensive ulcerative colitis (Lennard-Jones et al, 1983) or with autosomal dominant inherited disorders such as familial adenomatous polyposis (FAP), resulting from germline mutations in the adenomatous polyposis coli (APC) gene (Groden et al, 1991), or the hereditary non-polyposis colorectal cancers (HNPCC), due to mutations in one of several DNA mismatch repair genes (Kinzler and Vogelstein, 1996), the aetiopathogenesis for the majority of sporadic cases remains poorly understood. Despite major advances in the early diagnosis, either as screening tests for faecal occult blood, or more recently by endoscopy, and in the treatment of colorectal cancer, there has been no significant improvement in reducing mortality over
the last two decades with overall five year survival remaining at 35%. With a poor prognosis due to the late stage of presentation of the tumour, prevention is therefore the key to survival. One route of primary or secondary prevention would be the refinement of molecular screening methods designed to focus attention on individuals at higher risk due to their genetic makeup. However these methods cannot be applied to the common sporadic variety of colorectal cancer.

The developmental path for most colorectal cancers is well documented. Malignant tumours arise from pre-existing benign tumours over a period of years or even decades (Montoya and Wargovich, 1997). Histopathologically it starts with cellular hyperproliferation in the colonic mucosa, eventually leading to formation of adenomas with varying degrees of malignant potential and finally adenocarcinoma (Fearon and Vogelstein, 1990, Hamilton, 1992). A new dimension in the management of carcinogenesis is the increased awareness that chemoprevention, an area of cancer prevention research that recognises carcinogenesis to be an evolving multi-step molecular and cellular process (Greenwald et al, 1995), may be a way to reduce the risk of colon carcinogenesis. Increasing efforts are being made towards developing more effective prevention and screening measures. In chemoprevention, non-cytotoxic nutrients or pharmacological agents that protect against the development and progression of mutant clones or malignant cells, are used to inhibit or reverse the carcinogenic process, before malignancy (Kelloff et al, 1994). The suggestion that colon cancer is amenable to chemoprevention is supported by a significant number of studies using colon cancer cells in culture, carcinogen-induced tumours in mice and rats, and the growth of human xenographs in nude mice (Lipkin, 1992). In the search for new cancer chemopreventive agents over the past few years, hundreds of plant extracts have been evaluated (Sharma et al, 1994). A number of micronutrients, macronutrients and other dietary agents have been reported as having inhibiting or chemopreventive effects in chemical carcinogenesis models in rodents. At the root of these studies lies the concept of multistage carcinogenesis. An understanding of the mechanisms involved in the process of colon cancer is critical to the development of chemopreventive agents that inhibit stages along the malignant pathway (Kelloff et al, 1994). Chemopreventive agents can then be characterised according to the stage that they inhibit. Prevention of colon cancer demands the development of biomarkers that can assist in the rapid evaluation of potential chemopreventive agents and of nutritional agents, for their utility
in clinical chemoprevention trials. The intermediate biomarker should be closely associated with the causal pathway for carcinogenesis, for example proliferative signals and genetic changes, such as DNA content and oncogene and tumour suppressor gene expression.

An understanding of the molecular genetics of colorectal cancer has only recently been investigated. Because colorectal tumours evolve through well defined morphological stages, it has been possible to approximate the order in which gene alterations may occur (Kinzler and Vogelstein, 1996). The accumulation of genetic alterations appears to be more important than a particular sequence of gene inactivation (Fearon and Vogelstein, 1990). Recent measurements of cell proliferation, differentiation and gene structure have identified abnormal stages of cell development that are associated with an increased susceptibility to cancer.

Experimental studies in animal carcinogenicity models have demonstrated that at least three stages can be distinguished in colorectal cancer; initiation, promotion and progression. Initiation is the irreversible alteration in the genetic structure of a cell that confers the potential for a selective clonal expansion compared to the surrounding normal cells, leading to the development of a neoplastic clone of cells (Wright et al, 1994). Modifications to DNA include gene mutations and DNA rearrangements. Cell proliferation then fixes the modified DNA in initiated cells. An initiated cell may remain dormant for a long time until exposure to a promoting agent. Further cell proliferation and events involved in tumour promotion leads to the clonal expansion of those cells containing altered DNA, and subsequent growth of the nascent tumour (Wright et al, 1994). Tumour promotion, as well as involving stimulation of proliferation, is accompanied by the accumulation of structural gene alterations and altered gene expression, in particular genes that play a role in growth and differentiation. It also involves inflammatory changes and enzymatic changes, for example changes to ornithine decarboxylase (ODC), protein kinase C (PKC), and enzymes of the arachidonic acid cascade (i.e. interactions with pathways of intracellular signal transduction). Transformation of colorectal epithelium to adenomas and carcinomas has been shown to be associated with a progressive inhibition of apoptosis (Payne et al, 1995; Bedi et al, 1995), in cells that ordinarily would be programmed to die but may have undergone carcinogenic mutations that provide extended life span.
Hence inhibition of apoptosis in colon cancer may contribute to tumour growth and promote neoplastic progression. Therefore chemopreventive agents may not only decrease cell proliferation, but may also increase apoptosis.

The existence of multiple genetic alterations in human colon cancer has been demonstrated by Fearon and Vogelstein (1990) who have developed a preliminary genetic model for colorectal tumourigenesis. The model for colon tumourigenesis is shown in Figure 1.1. Genetic changes that occur during colon carcinogenesis include mutations in critical genes such as K-ras, APC, deleted in colon cancer (DCC), mutated in colon cancer (MCC) and p53 (Marx, 1989). The combined effects of the mutational activation of oncogenes, the activity of which leads to enhanced growth, and the inactivation of several tumour suppressor genes, may play a significant role in colon tumour development (Vogelstein and Kinzler, 1993). Progression to a more malignant phenotype (ie transition of normal colorectal epithelium to adenomas and their malignant transformation to adenocarcinomas) is accompanied by a progressive increase in the number of genetic alterations (Sugimura, 1992). Although genetic alterations appear to act at specific stages during colon tumour progression it is the accumulation rather than the order of genetic alterations, that appears to be most important (Fearon and Vogelstein, 1990). The molecular events that bring about such genetic mutations are possible targets for the chemoprevention of neoplastic progression in colorectal carcinogenesis (Greenwald et al, 1995).

Epidemiological and laboratory studies indicate that dietary factors play a significant role in the etiology of colon cancer (Willett et al, 1990). Doll and Peto (1981) suggested that dietary modifications could reduce the death rate due to colon cancer by as much as 90%. However the etiology of colon cancer is multifactorial and complex in that it may arise from the combined actions of low level environmental factors, genotoxic agents, and dietary and host factors, such as high fat diets. Tumours of the large bowel are 100 times more frequent than cancers of the small intestine, suggesting that dietary components undergo metabolic activation after absorption from the small intestine, or are formed endogenously after passage into the colon (Van Tassell et al, 1990). The role of these environmental and dietary factors, as possible modifiers contributing to an increased risk of human colon cancer is of great importance. Identification of naturally occurring dietary carcinogens and anti-carcinogens, may lead to new strategies for
Figure 1.1: Mutational events involved in the multistage model of human colon carcinogenesis. Sequential loss or deletion of sections of specific chromosomes 5, 17 and 18 results in loss of heterozygosity of the genome. If the remaining allele is inactivated by somatic or inherited mutation, then loss of tumour suppressor activity may occur. These events coupled with with a mutational activation of a protooncogene (K-ras), may result in tumour formation. The accumulation of genetic and epigenetic changes appears to be more important than their sequence.

From Fearon and Vogelstein (1990)
cancer prevention, and may allow study into the mechanisms of carcinogenesis. It has been suggested that targeting the stage of promotion has the greatest potential for cancer prevention, whereas cancer therapy is generally administered during the stage of progression (Samaha et al, 1997). The use of naturally occurring substances that are derived from the diet for chemoprevention, provides a strategy to inhibit cancer and these agents should have low toxicity towards normal cells.

1.2: Endogenous tumour promoters relevant to human colon carcinogenesis

1.2.1: Fecapentaenes

Faecal mutagenicity has been shown to be a risk factor in colon cancer (Bruce et al, 1977). Fecapentaenes form the group of most prevalent genotoxins excreted by the majority of human individuals in Western populations (Schiffman et al, 1989a), and were first described in 1977 by Bruce and co-workers. The fecapentaene concentration in human stool samples has been calculated to be between 0.3 and 30µM (Schiffman et al, 1989a). Since fecapentaenes are lipophilic and therefore readily absorbed by cells, it is possible that the above concentration could occur in vivo in human colon epithelial cells (Plummer et al, 1995). FP-12 is a commercially available model fecapentaene, and the most abundant form found in faecal extracts, accounting for approximately 60% of the total fecapentaenes found in human stool extracts (de Kok et al, 1992). The structure of FP-12 was reported by Bruce et al (1982). FP-12 is a 1-(1-glycero) dodeca-1,3,5,7,9-pentaene, and is characterised by the presence of a conjugated linear hydrocarbon chain consisting of five double bonds linked to a glycerol moiety via an ether bond. The structure of FP-12 is shown in Figure 1.2. FP-12 has a specific ultraviolet (UV)-triplet absorbance spectrum, exhibiting UV maxima at 323, 338 and 357nm (Gupta et al, 1983), is highly unstable and undergoes degradation when exposed to light, oxygen or acidic pH (Hirai et al, 1982; Dion and Bruce, 1983).

Fecapentaenes are synthesised by various Bacteroides species in the human colon from polyunsaturated ether phospholipid precursors of unknown origin called plasmalopentaenes (van Tassell et al, 1989; Kingston et al, 1989). The sources of fecapentaenes are likely to be from the diet, colonic microfloral cell membranes or host cells (Povey et al, 1991). Vegetarians, who have a decreased risk of colon cancer
compared to omnivores, excrete higher amounts of FP-12 than omnivores (de Kok et al, 1992). Colon cancer patients absorb more fecapentaenes, which leads to smaller amounts being excreted than controls (Schiffman et al, 1989b). These inverse relationships between FP-12 excretion levels and risk of colorectal neoplasia, suggest that excretion of these compounds, as assessed by measurement of faecal concentration, reduces endogenous exposure of the colonic epithelium, thereby indicating a protective effect of high excretion rates, rather than marking a risk for the development of colon cancer.

Initial epidemiological studies have associated increased faecal mutagenicity, and by inference fecapentaenes, with increased colon cancer risk (Povey et al, 1991). FP-12 has been found to be a potent genotoxin in mammalian cells (Curren et al, 1987) and has been suggested to act as an initiating agent. Fecapentaenes account for approximately 90% of the direct acting mutagenicity of human faeces (Schiffman et al, 1989a), and have been reported to be 900 times more potent than N-methyl-N-nitrosourea, on a molar basis, in a human fibroblast hypoxanthine guanine phosphoribosyl transferase mutation assay (Plummer et al, 1986). In vivo studies have shown that FP-12 induces DNA single strand breaks and unscheduled DNA synthesis in the colon after administration intra-rectally (Hinzman et al, 1987).

![Figure 1.2: The structure of FP-12](image-url)
Studies on the mechanisms of genotoxicity, have indicated two different modes of interaction of FP-12 with DNA. The first mechanism involves a direct interaction, FP-12 acting as an alkylating agent, resulting in the formation of bulky DNA adducts, in \(^{32}\)P post-labelling experiments (Povey et al, 1992). The second mechanism proposed for FP-12 genotoxicity suggested an indirect mechanism, via the generation of reactive oxygen species (ROS). Increased mutagenicity in the Ames test using the *Salmonella Typhimurium* strain TA104, designed for its sensitivity to detect oxidative mutagens, indicated the involvement of ROS. Formation of oxygen radicals, such as oxy-radicals, hydroxyl radicals, or aldehydes indicated that FP-12 undergoes a lipid peroxidation type process resulting in its decomposition (Povey et al, 1991). These radicals can then interact with DNA, and cause oxidative base damage, based on evidence, such as the formation of 8-hydroxydeoxyguanosine (8-OHdG) in cellular DNA, during incubations of Hela cells with FP-12 (Plummer and Faux, 1994). This is supported by an *in vitro* study showing a dose response relationship between exposure of isolated DNA solutions to FP-12, and the formation of 8-OHdG adducts (Shioya et al, 1989). Oxy radical formation is further supported by the formation of small amounts of oxidised glutathione when reduced glutathione is treated with FP-12 (Dypbukt et al, 1989).

Based on these findings of pronounced *in vitro* genotoxicity, fecapentaenes were suspected of playing a causative role in human colon carcinogenesis. Subsequent *in vivo* studies using rodents, exposed intra-rectally to FP-12, failed to induce tumours (Ward et al, 1988, Weisburger et al, 1990), suggesting FP-12 has weak carcinogenic activity in animal models.

Because of its mutagenic activity, it might be assumed that if FP-12 does play a role in colon carcinogenesis it would be due to its genotoxic effects and therefore might act at the stage of initiation or progression. However, Hoshina et al (1991) suggested that FP-12 could act as a tumour promoter by enhancing PKC activity in the colon epithelium. They suggested that fecapentaenes mimic 12-O-tetradecanoyl phorbol-13-acetate (TPA) by substituting for endogenous phospholipid cofactors to stimulate PKC activation. FP-12 has recently been shown to possess tumour promoting activity in a rat colon carcinogenesis model system, using N-methyl-N-nitrosourea as the initiating agent (Zarkovic et al, 1993). Exogenous oxidants and agents which induce a cellular pro-oxidant state, such as the classical tumour promoter TPA, have been implicated in tumour promotion (Crawford et al, 1988). There is evidence to suggest that FP-12 could
act at least in part through the production of ROS, producing “oxidative stress”, in cells, but its importance in tumour promoting activity, at the level of early response gene transcription, remains to be determined. Oxidative stress is due to chronic exposure to reactive oxygen species in the form of singlet oxygen, superoxide radicals, \( \text{H}_2\text{O}_2 \) and hydroxyl radicals (Cerruti and Trump, 1991). Understanding the mechanisms involved in FP-12 induced tumour promotion may help to find agents that could block the promotional activity of FP-12.

1.2.2: TNFα

TNFα, a protein with a molecular weight of 17,000 Da, is a potent multifunctional, inflammatory cytokine, and is synthesised by a wide range of cells, including macrophages, T and B lymphocytes, natural killer cells, some transformed cell lines and breast and epithelial tumour cells (Fiers, 1991). Macrophages, activated by certain stimuli such as endogenous cytokines and bacterial products, such as lipopolysaccharide (LPS), are considered to be the main producer of TNFα in vivo, (Vilcek and Lee, 1991). It was initially described in serum of endotoxin-treated mice as the mediator of the necrosis of some transplantable tumours (Old, 1985). TNFα has since been reported to be cytotoxic to certain transformed cell lines, and may act as a mediator in beneficial processes of host defence, immunity, and tissue homeostasis, or in the pathogenesis of infection, tissue injury and inflammation (Tracey et al, 1989).

TNFα has been shown, in vitro, to be involved in mediating a wide range of biological activities (Fiers, 1995) and has been found to be a potent mediator of the inflammatory process. Inflammation as a general consequence of TNFα action is likely to be related to TNFα stimulation of arachidonic acid release (Atkinson et al, 1990), activation of cytosolic phospholipase A2 (PLA2) and production of pro-inflammatory mediators like prostaglandins and leukotrienes (Heller and Kronke, 1994). TNFα is also involved in mediating differentiation of immature cell lines, antiviral responses and can either enhance or inhibit cell proliferation in various cell lines (Kaiser and Polk, 1997). TNFα stimulates proliferation of many cell types, both lymphoid and non-lymphoid, including thymocytes (Ranges et al, 1988), fibroblasts (Sugarman et al, 1985), chondrocytes (Ikebe et al, 1988), mouse colon epithelial cells (Kaiser and Polk, 1997) and some human cancers, including chronic lymphoid leukemia (Digel et al, 1984) and ovarian
cancer (Naylor et al, 1993). TNFα can also mediate apoptotic or necrotic cell lysis (Laster et al, 1988).

The fact that TNFα can activate both proliferative and apoptotic signals, may be due to TNFα receptor binding. TNFα exerts its biological effects, through binding to two cell surface receptors; one is ~55kD (p55 tumour necrosis factor receptor (TNFR) or TNFR1) and the other is 70-80kD in size (p75 TNFR or TNFR2) (Loetscher et al, 1990; Schall et al, 1990; Vandenabeele et al, 1995). The receptors are glycoproteins with a single membrane-spanning hydrophobic segment, and they are expressed at the surface of most cells, although in varying amounts. Most of the known TNFα responses occur by the activation of the p55 TNFR (Wiegmann et al, 1992). The binding of TNFα to its receptors leads to the activation of several second messengers, although the response is often cell type specific (Beyaert and Fiers, 1994). Once TNFα binds to receptors on cells, its intracellular signalling pathways are complex. Potential signal transduction events that occur following stimulation with TNFα include activation of multiple protein kinases, including Jun N-terminal kinase/ stress activated protein kinase (JNK) (Modur, 1996; Liu et al, 1996) protein kinase A (PKA) (Zhang et al, 1988) and PKC, (Schutze et al, 1989) phospholipases, (Kaiser and Polk, 1997) p38 mitogen activated protein kinase (MAPK), (Beyaert et al, 1996) and activation of a phosphatidylcholine specific phospholipase C, which in turn promotes the breakdown of sphingomyelin (Schutte et al, 1992a). TNFα has been reported to activate the recently identified IkB kinase (DiDonato et al, 1997). TNFα can also generate ROS (Schulze-Osthoff et al, 1992), activate phosphorylation and alter calcium homeostasis. By interacting with TNFR1, TNFα activates the transcription factors, nuclear factor-kappa B (NF-κB) and activator protein-1 (AP1), leading to the induction of proinflammatory genes (Brenner et al, 1989; Osbom et al, 1989; Claudio et al, 1996). TNFα has been shown to stimulate the transcription of a number of genes, including those encoding for c-fos, c-jun, c-myc and interleukin-6 (IL-6) (Kohase et al, 1986). One TNFα signalling pathway can initiate a death program, via interaction of the TNFα receptor complex with the Fas associated protein death domain (FADD) while another leads to a protective pathway, that involves activation of NF-κB (van Antwerp et al, 1996). There is increasing evidence that the induction of many TNFα responsive genes is mediated at least in part through the
activation of NF-κB (Schutze et al, 1995), by inducing IκBα phosphorylation and its subsequent degradation (Beg et al, 1993).

Although TNFα is one of the inflammatory cytokines, TNFα may play a part in various pathological states, such as in rheumatoid arthritis, Crohn’s disease, multiple sclerosis, HIV replication, malaria (Fiers, 1991) and is assumed to play a pivotal role in the process of human carcinogenesis (Suganuma, et al, 1996), probably as an endogenous tumour promoter. TNFα acts as an endogenous tumour promoter when it allows the clonal growth of initiated cells (Komori et al, 1993). The luminal concentration of TNFα is found to be elevated in patients with inflammatory bowel disease (Braegger et al, 1992), a condition which is associated with an increased risk of colon carcinogenesis (Gyde et al, 1988). TNFα is found in fresh human tissues, taken from patients, and can enhance metastasis (Gelin et al, 1991). Much attention has been focused on regulating the expression or inhibiting the activity of TNFα. Suganuma et al (1996) reported that inhibition of TNFα mRNA expression, resulting in inhibition of TNFα release is a common mechanism for cancer prevention.

1.3: Prostaglandin H synthase, as a target for tumour promoters

Prostaglandins, made by the enzyme PGHS, may have an important role in colon carcinogenesis (Reddy et al, 1992; Kutchera et al, 1996), by their growth promoting effects on colon tumour cells (Sheng et al, 1998). For example prostaglandin E2 (PGE2) can affect cell proliferation and inhibit the immune response to malignant cells; therefore overproduction of prostaglandins could favour malignant growth (Marnett, 1992). More than twenty years ago, high concentrations of prostaglandins were found in human and animal tissues. Reports have noted elevated levels of prostaglandins, such as PGE2 in various cancers including elevated levels, in colorectal adenomatous and carcinoma tissues, compared to normal mucosa (Marnett, 1992; Rigas et al, 1993; Puge and Thomas 1994). Additionally in tumour models, such as phorbol ester-dependent hyperplasia of mouse skin, a role for prostaglandins in tumour promotion has been reported (Furstenberger et al, 1989). Since it is known that prostaglandin synthesis is enhanced in cells by tumour promoters (Marnett, 1992) another pathway that may play
a part in the promotional effects of TNFα or FP-12 is that involving activation of COX-2 increasing the production of prostaglandins from arachidonic acid.

1.3.1: Two isoforms of PGHS

PGHS is a membrane bound hemoprotein that is widely distributed in mammalian tissues, but is not present in all tissues. There are two isoforms of the enzyme PGHS, in humans, cyclooxygenase-1 (COX-1) and COX-2 (Hla and Neilson, 1992; Jones et al, 1993). COX-1 was initially purified from bull vesicular glands by Miyamoto et al (1976) followed by Hemler and Lands (1976). The isolation and sequence analysis of cDNAs encoding ovine COX-1 was cloned and analysed from seminal vesicles in 1988 (DeWitt and Smith, 1988). Subsequent cloning of murine and human (Funk et al, 1991) COX-1 showed a 90% identical amino acid sequence among these species (DeWitt et al, 1990). The existence of a second isoform of PGHS was suggested after investigators studying cell growth signalling pathways, identified an inducible gene product related to the known COX sequence (Hershman, 1996). Investigators looking at prostaglandin production in response to cytokines and other inflammatory agents, observed increases in COX activity that could only be accounted for by increased expression of another cyclooxygenase (Raz et al, 1988). Immunoprecipitation of this COX variant with a COX-1 antibody, as well as the production of an antibody that only precipitated the COX-2 isoform, identified two different COX isoforms. COX-2 was cloned in 1991, by two independent groups, confirming the existence of two enzymes (Kujubu et al, 1991; Xie et al, 1991). COX-2 was identified as a primary response gene, TIS10 whose complementary deoxyribonucleic acid (cDNA) was cloned as a result of its rapid inducible expression in Swiss 3T3 cells in response to TPA (Kujubu et al, 1991). Independently, Xie et al (1991) found in chick embryo fibroblasts transformed with a temperature sensitive Rous Sarcoma virus (RSV) mutant, a 4.1kB messenger ribonucleic acid (mRNA) that encoded a protein containing about 60% amino acid homology with sheep COX-1.

COX-1 is a well characterised enzyme and is expressed constitutively in almost all tissues (O’Neill and Ford-Hutchinson, 1993; Crofford, 1997), but at different levels in various cell types, and is associated with the endoplasmic reticulum (Morita et al, 1995). COX-1 is considered to be a housekeeping gene (Funk et al, 1991) and
prostanoids synthesised via the COX-1 pathway are thought to be responsible for cytoprotection of the stomach, vasodilation in the kidney and production of thromboxane by platelets. COX-2, however, is not constitutive in most tissues, and COX-2 message and protein are normally undetectable (Langenbach et al, 1995), but can be induced in a variety of cells by diverse stimuli. COX-2 can be induced in vivo, during the inflammatory response (DuBois et al, 1994), and in vitro by growth factors (Hamasaki et al, 1993), cytokines, such as TNFα and interleukin 1 (IL-1) (Lyons-Giordano et al, 1993; Ristimaki et al, 1994), inflammatory stimuli, oncogenes and tumour promoters, such as phorbol esters (Kujubu and Hershman, 1992; Williams and DuBois, 1996). Expression of COX-2 has been shown in various cell types, including fibroblasts, mononuclear phagocytes, endothelial cells, neurons and in human articular chondrocytes (Geng et al, 1995). Human tissues including lung, uterus, testis, brain, pancreas, kidney, liver, thymus, prostate, mammary gland, stomach and small intestine all express detectable COX-2 mRNA, even without stimulation (O’Neill and Ford-Hutchinson, 1993). Constitutive COX-2 activity has been observed in several tissues and organs including kidney (Harris et al, 1994), brain and tracheal epithelial cells (Walenga et al, 1996) and in gastric mucosa (Zimmerman et al, 1998). COX-2 is primarily associated with the nuclear envelope, suggesting a direct effect on nuclear events (Morita et al, 1995).

The two isoforms are approximately 60% identical at the amino acid level, while the residues critical for enzyme function are highly conserved (Appleby et al, 1994), and therefore COX-2 contains all the structural features important for PGHS enzyme activity. However the COX-2 isoform is encoded by a distinct gene (Subbaramaiah et al, 1997). The mRNA transcript of the COX-2 gene is about 4.5 kb in length, compared to 2.8 kb for COX-1 and the regions regulating gene expression of COX-1 and COX-2 show little similarity (Kosaka et al, 1994). The COX-2 gene contains 10 exons in about 8kb instead of 11 exons in 22kb for COX-1 (Xie et al, 1993). This relatively small genomic size for the COX-2 gene is characteristic of an early response gene (Herschman, 1996). The promoter region regulating COX-2 contains various response elements that have been shown to explain at least in part, its inducibility by hormones, growth factors, and cytokines (DuBois et al, 1998). The elements involved in controlling the expression of the COX-1 gene are less well known. However the COX-1
and COX-2 genes are regulated by independent systems, even though the enzymatic reaction they catalyse is identical.

1.3.2: Enzyme activity of PGHS

Cyclooxygenase is the rate limiting enzyme for the biosynthesis of prostaglandins and thromboxane A2 (TXA2) from arachidonic acid (DeWitt, 1991), or other twenty carbon fatty acids. Arachidonic acid is derived directly from the diet or via modification of linoleic acid (Eberhart and DuBois, 1995). Prostaglandin synthesis is initiated by release of arachidonic acid from cell membrane phospholipids, by phospholipase A2 (PLA2) (Dennis et al, 1991). Cyclooxygenases (COX-1 and COX-2) have two distinct activities (Muller-Decker et al, 1995); as a cyclooxygenase in the oxidation of arachidonic acid, using two molecules of O₂ (Marnett, 1992), to form the hydroperoxide prostaglandin G2 (PGG2), and as a peroxidase in the conversion of PGG2 to prostaglandin H2 (PGH2) (Smith et al, 1991a). This is shown in Figure 1.3.

PGH2, produced by both COX-1 and COX-2, is a precursor for biologically important prostanoids, such as thromboxane, prostacyclin and PGE2 (DuBois et al, 1998). Specific synthases catalyse the conversion of PGH2, and the array of prostaglandins produced in a given tissue varies depending on which of these enzymes is expressed in a particular cell type (Eberhart and DuBois, 1995). For example vascular endothelial cells primarily produce prostaglandin I2 (PGI2), which is an inhibitor of platelet aggregation and a vasodilator, whereas platelets mainly produce TXA2, which is a potent proaggregatory and vasoconstrictive agent (Arita et al, 1989). A synthase which catalyses the production of PGE2 has recently been cloned (Jakobsson et al, 1999). Alternatively PGH2 can break down to a direct mutagen, malondialdehyde (Prescott and White, 1996), which can form adducts with deoxynucleosides (Basu et al, 1988).

Aside from being important for prostaglandin synthesis, PGHS has been implicated via its peroxidase activity, as a source of carcinogen activation in extra hepatic tissues (since it requires a cofactor as a reductant), especially in human colon carcinogenesis (Eling et al, 1990), but does not effect systemic xenobiotic oxidation. It has been reported (Plummer and Faux, 1994) that fecapentaenes are co-oxidised either directly or indirectly, by PGHS to intermediates that degrade to form ROS which cause oxidative
Figure 1.3: Arachidonic acid metabolic pathway for prostaglandin synthesis by COX-1 and COX-2. The arachidonic acid pathway of prostaglandin synthesis involving the conversion of arachidonic acid into prostaglandin G2 (PGG2), is mediated by COX-1 and COX-2. PGG2 is then converted into PGH2, and eventually into a number of other metabolites, including prostaglandins PGF2α, PGD2, PGE2 and TXA2. Traditional NSAIDs, such as aspirin, inhibit COX-1 and COX-2, whereas the new generation of NSAIDs selectively inhibit COX-2.
DNA damage. The cytochrome P-450 family of enzymes is widely recognised for catalysing oxidative reactions that convert xenobiotics to reactive electrophiles (Eling et al, 1990). Many xenobiotic compounds, including several classes of chemical carcinogens and heterocyclic aromatic amines, can be oxidised by cyclooxygenase to mutagens (Marnett, 1992). The resultant oxidised amines can react with DNA to produce adducts that may mutate critical tumour suppressor genes and lead to the initiation of the neoplastic process (Smith et al, 1991b). The potential importance of this mechanism is highlighted by the recent observation that benzo (a) pyrene-diol epoxide, a mutagen formed by COX, causes adducts along exons of the p53 gene that correspond to p53 mutational hotspots in lung cancer (Denissenko et al, 1996). The relative importance of the two PGHS isoforms in the metabolic activation of fecapentaenes and other carcinogens is not known. However Singh et al (1997) suggested that COX-2 may have an important role in the activation of carcinogens by converting carcinogens to reactive intermediates that mutate DNA. In addition to catalysing oxidation reactions to produce mutagens, COX catalyses reactions that may predispose to carcinogenesis via other mechanisms. Prostaglandin synthesis by COX may impair immune surveillance and killing of malignant cells (Subbaramaiah et al, 1997).

Although the key step in prostaglandin production is catalysed by COX-1 and COX-2 in the same catalytic reaction, these isoforms appear to mediate different biological functions (Lagenbach et al, 1995; Morham et al, 1995). It has been proposed that COX-1 is associated with the production of prostaglandins involved in cellular 'housekeeping' functions (Hershman, 1994). COX-1 is responsible for the production of prostaglandins required for gastrointestinal, renal and vascular homeostasis. An important site of COX-1 function is the blood platelet, where the enzyme provides precursors for thromboxane synthesis (Schafer, 1995). COX-2 however, is involved with various biological events such as injury, inflammation and proliferation (Masferrer et al, 1994). A recent paper has also suggested an anti-inflammatory role for COX-2 (Gilroy et al, 1999).
1.3.3: Importance of COX-2 in colon carcinogenesis

There is now considerable evidence from several different experimental systems that COX-2 may play a role in colon carcinogenesis in a causal way. COX-2 is not present in most tissues under physiological conditions, but is upregulated at an early (Hao et al, 1999), but as yet poorly defined stage during colorectal tumour formation. COX-2 but not COX-1 mRNA is elevated in human colorectal tumours, whereas the accompanying normal intestinal mucosa has low or undetectable COX-2 expression (Eberhart et al, 1994; Sano et al, 1995). This finding has been confirmed, since elevated levels of COX-2 protein has been seen in colorectal tumours by Western blotting (Kargman et al, 1995) and immunohistochemical staining (Sano et al, 1995). Kargman et al (1995) reported that 19 of 25 protein extracts from carcinomas were positive for COX-2 and in the same study 23 of 25 normal colonic tissue were negative for COX-2. Sano et al (1995) found by immunohistochemistry that COX-2 protein was markedly increased in tumour cells but not in the normal tissue, immediately adjacent to the tumour. Eberhart et al (1994) also found that a sub-set of colorectal adenomas, the precursor lesions in colorectal carcinogenesis showed upregulation of COX-2 mRNA. Studies have shown over-expression of COX-2 in about 90% of colorectal cancers (Kargman et al, 1995) and 40% of premalignant colorectal adenomas in humans, but is not expressed in non-tumour tissue (Sano et al, 1995). Patients with inflammatory bowel disease, a condition which predisposes to colon cancer show increased COX-2 mRNA expression indicating that COX-2 is involved in this disease (Hendel and Neilsen, 1997). In addition Yoshima et al (1997) observed over-expression of COX-2 mRNA in adenomas and carcinomas that develop in carcinogen treated rats, and in mouse adenomatous polyps. DuBois et al (1996) reported that COX-2 levels were dramatically increased in intestinal tumours which develop in AOM-treated Fischer-344 rats, but was absent in normal intestinal mucosa. Intestinal adenomas from Min mice, which have a germline mutation in the APC gene, and are an animal model for FAP, express increased levels of COX-2 mRNA and protein (Williams et al, 1996).

Genetic evidence has been reported to support a role of COX-2 in the development of colon carcinogenesis, which directly links COX-2 expression to intestinal tumourigenesis (Prescott and White, 1996). In the absence of COX-2, whether by pharmacological inhibition or genetic alteration, adenoma formation is impaired.
COX-2 gene knockout suppresses intestinal polyposis in APC<sup>Δ716</sup> knockout mice, a model system in which a targeted truncation deletion in the tumour suppressor gene APC causes intestinal polyposis (Oshima et al, 1996). In these studies mice carrying an APC mutation were mated with a mouse with a disrupted COX-2 gene. All of the animals were APC +/-; if homozygous for wild type COX-2, they developed an average of 652 polyps at 10 weeks, while heterozygotes had 224 polyps and homozygously deficient mice (COX-2 null mice) only 93 polyps. Tiano et al (1997) reported that COX-2 deficiency protected against the formation of extra-intestinal tumours. The COX-2 knockout mice developed about 75% fewer chemically induced skin papillomas than control mice. These experiments provided definite genetic evidence that induction of COX-2 is an early, rate limiting step in adenoma formation.

Further supporting evidence that COX-2 plays an important role in the pathogenesis of colorectal cancer is that NSAIDs protect against colon carcinogenesis in animal models, for example tumour multiplicity is dramatically decreased in Min mice treated with the NSAID, sulindac (Boolbol et al, 1996). Epidemiological studies suggest they may also protect humans from this disease (Thun et al, 1991; Rosenberg et al, 1991). Clinical studies of patients with FAP, an autosomal dominant disorder, in which almost all of those affected will develop colorectal cancer, by the fifth decade of life, if left untreated, indicated that administration of sulindac caused a reduction in polyps (Giardiello et al, 1993). NSAIDs inhibit directly the activity of both isozymes (COX-1 and COX-2) and reduce prostaglandin production (Meade et al, 1993). This property, that accounts for their shared therapeutic and side effect profiles, which include nephrotoxicity, gastrointestinal ulceration and bleeding, are due to blocking COX-1 activity (Eberhart and DuBois, 1995). These observations suggest that COX-2 promotes tumour development in gastro-intestinal tissue, and COX-2 could be an attractive target for the chemopreventive action of NSAIDs. In the study described in the previous paragraph by Oshima et al (1996) a novel selective inhibitor of COX-2, ‘Merck Frosst tricyclic’, markedly reduced the number of polyps in the APC<sup>Δ716</sup> mice, more significantly than the non-selective NSAID, sulindac.

Despite the many studies that correlate increased expression of COX-2 with colon cancer development, the molecular mechanisms underlying the contribution of COX-2 to carcinogenesis are still not clear. Several mechanisms could account for the link
between the activity of COX-2 and carcinogenesis, for example increased synthesis of prostaglandins can enhance proliferation (Tsujii et al, 1998), and inhibit immune surveillance (Huang et al, 1998). It has been suggested that COX-2 may be playing a role in cell growth and progression in colon cancer (Williams and DuBois, 1996). As prostaglandins, such as PGE2 produced by COX-2 have been shown to modulate cell proliferation in rat colonic epithelium, inhibition of COX-2 expression would be expected to have an effect on cell proliferation. Sheng et al (1997) suggested a direct link between inhibition of intestinal cancer growth and selective inhibition of the COX-2 pathway. However, Hao et al (1999) found no correlation between COX-2 expression and proliferation rates in human adenomas or carcinomas. Cell culture models have shown that COX-2 over-expression contributes to the tumourigenic potential of colonic epithelial cells, and recent experiments have suggested that COX-2 may modulate apoptosis (Prescott and White, 1996). Overexpression of the COX-2 gene in rat intestinal epithelial cell lines results in a delay in the cell cycle at the G1 phase, increased adhesion to the extracellular matrix, and resistance to sodium butyrate-induced apoptosis, possibly mediated by Bcl-2 and downregulation of E-cadherin (a cell adhesion protein) (Tsujii and DuBois, 1995). This could increase the tumourigenic potential of initiated cells. Ballif et al (1996) reported that COX-2 may modulate apoptosis by interacting directly with the apoptosis associated protein (Nuc) retaining it in the endoplasmic reticulum. The effects of COX-2 on apoptosis might also be indirect via the modulation of intracellular arachidonic acid as suggested by Chan et al (1998). However it is unclear what effects are involved in the ability of COX-2 to regulate cellular proliferation and apoptosis, in human colon epithelial cells.

In intestinal mucosa, in vivo, continuously proliferating stem cells are located in the crypt region (DuBois et al, 1996). Cellular differentiation and proliferative arrest are induced as intestinal stem cells ascend upward on the basement membrane along the crypt - villus axis toward the gut lumen (Tsujji and DuBois, 1995). When the cells reach the surface of the villi, they become fully differentiated, apoptosis is activated, and the cells are released into the lumen. The entire lifetime of an epithelial cell in humans is only 4-5 days. If COX-2 overexpression increases the time required in the G1 phase of the cell cycle, and inhibits apoptosis, this could increase the time that abnormal cells (with a primary mutation) would spend in transit from the villus tip and increase the chance that a second mutation could occur. Prolonged survival of genetically modified,
abnormal cells can favour tumour progression, and facilitate the accumulation of sequential genetic mutations that would result in tumour promotion (Dubois et al, 1996). The number of colonocytes depends on the rate of proliferation and the rate of apoptosis (Levy, 1997). An abnormality in either of these can result in neoplasia. Resistance to apoptosis, due to COX-2 over-expression is therefore one possible mechanism of tumour promotion. Consequently compounds that can inhibit COX-2 may enhance processes such as apoptosis and immune surveillance, which tend to destroy the initiated cells.

Possible mechanisms of increased expression of COX-2 in colorectal carcinogenesis, include stabilisation of COX-2 mRNA and protein or enhanced transcription of the gene (Muller Decker et al, 1995). Elevated levels of COX-2 protein and mRNA levels have been detected in human adenocarcinomas (Eberhart et al, 1994; Kargman et al, 1995), which suggests that induction is via enhanced transcription of the gene and not by stabilisation of COX-2 mRNA. Kutchera et al (1996) showed that COX-2 is overexpressed transcriptionally in colorectal cancer. Since COX-2 mRNA expression, like \textit{c-fos} and \textit{c-jun}, can be rapidly induced by phorbol esters, serum, cytokines and growth factors, it has been characterised as an early response gene (Jones et al, 1993; Kujubu et al, 1993). Tumour promoters such as FP-12 and TNF\(
alpha\) could activate the COX-2 gene, as one possible mechanism whereby they exhibit tumour promoting effects. Increases in PGHS enzymatic activity with concomittant enhanced capacity of cells to produce prostaglandins may occur via an increase in transcription of the cyclooxygenase genes (DeWitt, 1991). Chemopreventive strategies have focused on inhibitors of PGHS enzyme activity, but compounds that suppress the expression of COX-2 may also have anti-cancer properties.
1.4: Inhibitors of PGHS

1.4.1: Conventional NSAIDs

In an attempt to prevent cancer considerable effort is being directed toward developing chemopreventive agents that inhibit the activity of COX-2 (Siebert et al, 1994). As mentioned in section 1.3.3, aspirin and other NSAIDs, such as indomethacin, piroxicam, sulindac and ibuprofen, that inhibit prostaglandin synthesis, have been reported to protect against the development of colorectal cancer (Thun et al, 1991, Rosenberg et al, 1991; Giardiello et al, 1995), and possibly cancer of the urinary tract, mammary gland, skin and liver. Several recent studies have shown that individuals regularly using aspirin, or another NSAID had a 40-50% decrease in mortality from colorectal cancer compared to those not taking these drugs (Kane et al, 1988; Rosenberg et al, 1991; Thun et al, 1991; Marnett, 1992; Thun et al, 1993), suggesting that these drugs serve as effective cancer chemopreventive agents. In addition, in clinical trials, sulindac caused regression of rectal polyps and pre-existing adenomas in patients with FAP (Labayle et al, 1991; Giardiello et al, 1993; Spagnesi et al, 1994). Studies in several animal models of colorectal carcinogenesis have shown that several NSAIDs exhibit chemopreventive effects, causing a reduction in the frequency and number of pre-malignant and malignant changes (Reddy et al, 1993; Rao et al, 1995). Carcinogen induced tumours are also markedly reduced in animals treated with NSAIDs (Reddy et al, 1993). Piroxicam markedly reduces the formation of spontaneous intestinal tumours in the APC min mouse (Jacoby et al, 1996). Some of the animal studies have shown as much as an 80-90% reduction in tumour burden due to NSAIDs (Kawamori et al, 1998).

In 1893 a German chemist, Felix Hoffman, in trying to help his father's severe rheumatoid arthritis, set into motion a commercial process to produce a molecule with analgesic activity called acetyl salicylic acid or aspirin. From this evolved the class of drugs NSAIDs, that are still amongst the most widely used therapeutic agents known to man 100 years later. NSAIDs have been widely utilised agents for the treatment of acute and chronic inflammatory disorders such as rheumatoid arthritis (Masferrer et al, 1994), pain and fever, some for over a century. Studies of the effect of NSAIDs on the risk of colorectal cancer initially evolved independently of experimental work on the mechanism of action of these drugs. However in 1971, Vane proposed that the ability of
NSAIDs to suppress inflammation rests primarily in their ability to inhibit the cyclooxygenase enzyme, thereby inhibiting the PGHS pathway of arachidonic acid metabolism. This would limit the number of pro-inflammatory prostaglandins at the site of injury. *In vitro* studies showed that the drugs in common clinical use at that time were either more selective for the inhibition of COX-1 or were COX-1 and COX-2 dual inhibitors (Meade et al, 1993). One mechanism by which NSAIDs may have chemopreventive properties, is by inhibiting COX-2 enzymatic activity (Sano et al, 1995).

Only the cyclooxygenase and not the peroxidase function of COX is the target for agents, such as aspirin and indomethacin (Mizuno et al, 1982, Marshall and Kulmacz, 1998), to which they bind irreversibly, which potentially limits their effectiveness. Down-regulation of COX-2 gene expression may be a better strategy because it inhibits both cyclooxygenase and peroxidase enzyme activities. Chronic use of these drugs has been linked to the induction of gastrointestinal mucosal lesions, perforations and bleeding, in part of the population, and nephrotoxicity (Eberhart and DuBois, 1995: Langenbach et al, 1995; Reddy et al, 1996). These limitations of NSAIDs have been associated with the inhibition of prostaglandins in the stomach and kidney (Masferrer et al, 1994), causing an imbalance of prostaglandins that are produced from COX-1 in these tissues since these drugs have no selectivity for inhibition of COX-1 or COX-2 activity. The ability of NSAIDs to inhibit COX-2 may explain their therapeutic efficacy as anti-inflammatory drugs, since this isoform is rapidly elevated during inflammation whereas inhibition of COX-1 may explain their unwanted side effects (Vane, 1994), since this isoform is necessary for cellular housekeeping. This has lead to investigations into differentiating between NSAIDs interactions with COX-1 or COX-2 enzymatic activities to see if it is possible that the therapeutic effect of NSAIDs could be separated from their toxic gastrointestinal effects.

Treatment with NSAIDs is associated with a decrease in COX-2 in colonic tumours (Boolbol et al, 1996). Recently a new class of aspirin-like drugs, the so called COX-2 selective NSAIDs have been developed. Agents that are specific inhibitors of COX-2 may exhibit fewer gastrointestinal side effects than conventional PGHS inhibitors, that inhibit both COX-1 and COX-2 (Masferrer et al, 1994; Marnett and Kalgutkar, 1998). The possibility that COX-2 selective inhibitors will spare renal function is less clear cut.
Therefore selective COX-2 inhibitors may improve chemoprevention at the level of tumour promotion. A selective COX-2 inhibitor should block prostaglandin production in inflammatory cells, whilst not interfering with the homeostatic production of prostaglandins in the gastrointestinal tract (Vane, 1994). In animal models COX-2 specific NSAIDs reduce inflammation without these side effects. BF-389 was one of the earliest inhibitors shown to have a selectivity for COX-2 and showed little or no gastric ulceration (Wong et al, 1992). Another selective COX-2 inhibitory agent is celecoxib (SC-58635) (Reddy et al, 1996), which has been shown to inhibit the formation of AOM induced aberrant crypt foci (ACF) formation in the colon of male F344 rats. Kawamori et al (1998) showed that feeding SC-58635 to rats completely prevented the development of chemically induced tumours in 93% of animals. Furthermore the degree of inhibition of colon carcinogenesis by celecoxib exceeded that seen with aspirin and sulindac. O-acetoxyphenyl hept-2-ynyl sulfide (APHS) is sixty times more effective at blocking COX-2 activity than aspirin and it irreversibly inhibits enzyme activity in macrophages and colon cancer cells. Another selective COX-2 inhibitor, NS-398, yielded anti-inflammatory, antipyretic and analgesic properties as potent as those of indomethacin, without any serious side effects (Futaki et al, 1993; Futaki et al, 1994). Yoshima et al (1997) reported that NS-398 inhibited the development of AOM-induced ACF in male F344 rats after oral administration. NS-398 caused a dose dependent anti-proliferative effect in HT-29 cells (Elder et al, 1998). However, these studies do not take into account drug effects on COX-1 or COX-2 expression at the mRNA and protein level. Clinical trials evaluating compounds that are specific inhibitors of COX-2 have commenced, and preliminary results indicate a paucity of gastrointestinal side effects (DuBois et al, 1998). At present only nimesulide, a sulfonanilide class NSAID is used clinically and is found to be less ulcerogenic but has more or less the same anti-inflammatory and analgesic properties as classical NSAIDs. It has been reported to possess chemopreventive potential against colon cancer in rodents (Nakatsugi et al, 1996).

Although a relationship between overexpression of COX-2 and induction of colorectal cancer is well supported, it is still unclear whether all NSAIDs act as chemopreventive agents through inhibition of COX-2 activity. Understanding the mechanisms by which NSAIDs affect colon epithelial cells is important for the development of effective chemopreventive strategies for colorectal cancer. The signal transduction pathways and
transcription factors that mediate the induction of COX-2 provide additional important targets for the selective down regulation of COX-2 by NSAIDs. Recent studies have shown that salicylate inhibits NF-κB and AP-1 mediated gene transcription (Kopp and Ghosh, 1994; Dong et al, 1997), although at concentrations greater than 5mM. Kopp and Ghosh (1994) reported that aspirin and salicylate inhibited LPS induced NF-κB activation by interfering with the pathway leading to the phosphorylation and degradation of IκB. Chen et al (1997) demonstrated that aspirin suppressed silica-induced NF-κB activation in rat alveolar macrophages, possibly by inhibiting IκBα. Recently Yin et al (1998) reported that aspirin and salicylate selectively inhibited IκB kinase β. In comparison Schwenger et al (1997) reported high concentrations of aspirin inhibited the activation of JNK. As there is no evidence that IKK-β is involved in activating JNK, this suggests that there is more than one target for aspirin.

The mechanism behind the chemopreventive effect of NSAIDs may be independent of prostaglandin production, since NSAIDs may exert their tumour inhibitory effects at the molecular level through inducing tumour cells to die by apoptosis (Keller et al, 1998). One NSAID, sulindac, was reported to increase the rate of programmed cell death in the colonic epithelium of FAP patients (Tsujii and Dubois, 1995). Pasricha et al (1995) demonstrated that a colonocyte cell suspension prepared from mucosa biopsies of FAP patients treated with sulindac, displayed higher levels of apoptosis relative to mucosa biopsies obtained from the patients prior to treatment. At extremely high concentrations NSAIDs have been reported to induce programmed cell death directly in cultured cells. Shiff et al (1995) showed that sulindac and its metabolite sulindac sulphide reduced cell proliferation and induced apoptosis in HT29 cells. Lu et al (1995) found NSAIDs induce apoptosis and inhibit proliferation of v-src-transformed chicken embryo fibroblasts. Apoptosis, and the relationship between cell division and cell death are believed to be crucial in tumourigenesis, and defective regulation of apoptosis can promote tumour development. Colorectal epithelial homeostasis is dependent not only on the rate of cell proliferation, but also on apoptosis (Pasricha et al, 1995). Some traditional and COX-2 selective NSAIDs inhibit the synthesis of prostaglandins, which upsets the balance between tumour cell proliferation and apoptosis (Sheng et al, 1998). Recent data suggests that inhibition of COX-1 and COX-2 by NSAIDs may induce cell death by increasing the cellular pool of arachidonic acid (Chan et al, 1998). This in turn stimulates the production of neutral sphingomyelinase, resulting in the hydrolysis of
sphingomyelin to ceramide, which promotes apoptosis. It is not clear whether COX-2 selective inhibitors also cause an increase in cellular arachidonic acid, and set off this cascade.

Other mechanisms of apoptosis induction have been suggested for NSAIDs, which appear to act independently of prostaglandin synthesis. Sodium salicylate and some other NSAIDs (e.g. indomethacin and sulindac sulfone) have induced apoptosis in colon cancer cells, despite a lack of cyclooxygenase inhibitory activity (Piazza et al., 1997). Animal studies have shown that traditional NSAIDs can induce apoptosis and are chemopreventive without the need to inhibit COX-2 (Hanif et al., 1996). Recent studies have clarified that sulindac sulfone, a metabolite of sulindac, does not inhibit COX-1 or COX-2, but restores normal levels of apoptosis (Piazza et al., 1995) in the colonic epithelium of Min mice, and therefore is acting through a non-COX target. Elder et al (1997) reported that a COX-2 selective inhibitor induced apoptosis in HT29 cells, a human colorectal carcinoma cell line, which were lacking detectable COX-2 protein expression. Indomethacin, sulindac and sulindac sulfide inhibited cell progression and caused HT29 colon cancer cells to accumulate during the G0/G1 phase of the cell cycle and induced cell death (Shiff et al., 1995). The effect of NSAIDs at high doses, are not likely to be related to the ability of these compounds to inhibit COX, because compounds that have no anti-inflammatory activity and do not inhibit COX-1 or COX-2 (e.g. sulindac sulfide and sulfone) induce apoptosis in HT29 cells when given at concentrations ranging from 0.5 to 0.75 mmol/l (Piazza et al., 1995).

Overall, the evidence suggests that NSAIDs are effective suppressants of tumour cell proliferation, can prevent or reduce the occurrence of experimentally induced colonic epithelial tumours, and that in humans use of NSAIDs for other therapeutic reasons, reduces the frequency of colorectal cancer. Side effects may preclude their use as chemopreventive agents. The problem with aspirin and other NSAIDs as chemopreventive agents is that when their potential chemopreventive benefits are weighed against the risk of potential side effects, it is evident that a recommendation for their widespread use at the usual therapeutic dose may never be justifiable (Trujillo et al., 1994).
1.4.2: Naturally occurring anti-inflammatory agents

1.4.2.1: Curcumin

As a result of side effects associated with COX-1 inhibition much research is being carried out to develop selective COX-2 inhibitors. Such agents could act by directly inhibiting the peroxidase or cyclooxygenase component of COX-2, and/or inhibiting COX-2 gene expression (Subbaramaiah et al, 1998). In searches for new cancer chemopreventive agents over the past several years, hundreds of diet derived plant compounds have been evaluated for their potential to inhibit COX. Most of these agents have been found to be weak direct COX inhibitors but can also act through inhibition of COX-2 expression. Isolation, identification and testing of active substances, not only provide naturally occurring novel agents as inhibitors of cancer development but also gives opportunities to study the mechanisms of carcinogenesis. Curcumin is one such agent, and is currently being investigated as a possible chemopreventive agent (Krishnaswamy, 1993).

The use of naturally derived medicinal plants or their crude extracts in the prevention and/or treatment of several chronic diseases has been traditionally practiced in various different ethnic groups worldwide. The powdered dry rhizome of the plant *Curcuma longa Linn* (turmeric) has been used as a naturally occurring medicine for the treatment of inflammatory diseases (Ammon and Wahl, 1991). Curcumin, a phenolic compound is the major yellow pigment present in turmeric (Sharma, 1976), and possesses both anti-inflammatory (Huang et al, 1991a) and antioxidant properties (Rao et al, 1995b; Ruby et al, 1995). Turmeric and curcumin have been used as food colouring agents and spices in many foods such as curry and mustard, for centuries in Southeast Asia (Govindarajan, 1980) as well as in cosmetics and drugs. Turmeric is also an approved food additive and is available commercially at a low price. Curcumin is composed of two ferulic acid moieties, joined by a methylene bridge (Tanaka et al, 1994). Figure 1.4A shows the structure of curcumin.

As an antioxidant, curcumin scavenges ROS such as hydroxyl radicals, superoxide anion radicals (Kuncchandy and Rao, 1990) and singlet oxygen. Curcumin also interferes with lipid peroxidation (Sreejayan and Rao, 1994), xanthine oxidase activity
Figure 1.4: The structures of curcumin, CAPE and resveratrol.
(Lin and Shih, 1994), nitric oxide synthase activity (Brouet et al, 1995) and nitrite/nitrogen oxide production (Chan et al, 1995) therefore acting to protect various cellular constituents, including DNA and protein, from oxidative injury. More importantly, as an anti-inflammatory agent, curcumin is a potent inhibitor of both epidermal lipoxygenase and cyclooxygenase activities (Huang et al, 1991), thereby suppressing prostaglandin synthesis. This observation suggests that inhibition of arachidonic acid metabolism, for example by inhibiting PLA2, by curcumin may be partly responsible for its chemopreventive effects. The effect of curcumin during the initiation phase of colorectal cancer is less well characterised than the promotion phase. Curcumin has been reported to be non-mutagenic and acts as a strong antimutagen in animals (Nagabhushan and Bhide, 1986). Curcumin has been shown to inhibit colon carcinogenesis during the postinitiation stage, through modulation of COX activity in tumour tissue (Rao et al, 1995b).

The potential of curcumin to be chemopreventive was first demonstrated in vitro using cultured rat hepatocytes, where curcumin protected against tetrachloromethane-induced toxicity (Kiso et al, 1983). Consistent with its anti-inflammatory and anti-oxidant activity, curcumin has been shown to have chemopreventive activity against chemical carcinogen induced tumour formation in animals (Azuine and Bhide, 1992) during both initiation and promotion stages (Huang et al, 1992; Rao et al, 1995b). Topical application of curcumin to skin, inhibited tumour initiation by 7,12-dimethylbenz[a]anthracene (DMBA) in female CD-1 mice (Huang et al, 1988). Rao et al (1993a) showed that 2000ppm curcumin in the diet, during the initiation and post-initiation period, significantly inhibited AOM induction of colonic ACF formation, a putative early preneoplastic lesion and colon tumour incidence and multiplicity in male F344 rats. In similar studies feeding of 2% curcumin during initiation/postinitiation periods, resulted in decreased numbers of AOM induced dysplasias and colon tumours in CF-1 mice (Huang et al, 1994). Pereira et al (1996) reported that administration of 0.8 and 1.6% curcumin continuously during the postinitiation phases significantly inhibited development of AOM induced colonic adenomas in rats. Curcumin is an effective inhibitor of tumour promotion in rodent skin, colon and stomach carcinogenesis (Huang et al, 1994). Studies have shown that topical application of curcumin inhibits TPA-induced epidermal DNA synthesis and tumour promotion in mouse skin (Huang et al, 1988), edema of mouse ears and hyperplasia. In another study dietary administration of
2% tumeric inhibited DMBA -induced skin and benzo[a]pyrene -induced fore-stomach tumours in mice (Huang et al, 1992). Curcumin has been shown to inhibit the development of chemically induced tumours of the oral cavity (Tanaka et al, 1994). Kawamori et al (1999) reported that curcumin has chemopreventive activity if administered prior to, during and after AOM treatment to F344 rats as well as given only during the promotion/progression phase of colon carcinogenesis. These studies show that curcumin can be protective in various cancer model systems.

There is evidence to suggest that curcumin acts on pathways that may inhibit cell proliferation and enhance apoptosis (Hanif et al, 1997). Curcumin may be a chemopreventive agent that inhibits the growth of tumour cells through blocking enzymes, such as serine/threonine protein kinases as well as protein tyrosine kinases (Chen and Huang, 1998) involved in mitogenic cellular signal transduction pathways. Several lines of evidence indicate that the mechanism of action of curcumin is not limited to prostaglandin inhibition. Curcumin can inhibit the proliferation, and induce cell cycle changes in immortalized mouse embryo fibroblast NIH 3T3 cells, human colon adenocarcinoma cell line HT-29 (Hanif et al, 1997), human kidney cell 293, human hepatoma HEPG2 cells and human umbilical vein endothelial cells (Singh et al, 1996) with various common features of apoptosis, independently of its affect on arachidonic acid metabolism by COX. Curcumin has been shown to induce apoptosis in human hepato-blastoma (Jiang et al, 1996) and promyelocytic leukemia HL60 cells (Kuo et al, 1996). Hanif et al (1997) reported that curcumin caused cell cycle arrest in G2/M. Chen and Huang (1998) reported that curcumin caused a G0/G1 cell cycle arrest and reduced c-myc and Bcl2 mRNA levels, in vascular smooth muscle cells.

The mechanism of action of curcumin is complex; it can inhibit several mediators and enzymes involved in multiple cell signal transduction pathways. Curcumin can inhibit intracellular calcium dependent endonuclease by calcium depletion, reduce p53 gene expression and finally induce HSP70 gene expression by increasing the binding activity of the Heat shock factor in COLO2 colon carcinoma cells (Chen et al, 1996). Curcumin may inhibit protein phosphorylation by blocking the activities of diverse protein kinases, such as PKC (Liu et al, 1993), and the receptor tyrosine protein kinases (Korutla et al, 1995). The ability of curcumin to inhibit the growth of mouse NIH 3T3 cells and A431 cells has been correlated with a decrease in epidermal growth factor
receptor phosphorylation and epidermal growth factor receptor kinase, respectively (Korutla and Kumar, 1994; Korutla et al, 1995). Curcumin inhibits the activity of several different serine/threonine and tyrosine kinases in vitro (Reddy and Aggarwal, 1994). Curcumin may suppress TPA induced lipid peroxidation and the formation of 8-OHdG in mouse fibroblast, NIH 3T3 cells by inhibiting the activity of xanthine oxidase (Shin and Lin, 1993; Lin and Shih, 1994). Curcumin inhibits polyamine formation by blocking the activity of ODC (Lu et al, 1993; Rao et al, 1993a). Curcumin also inhibits TNFα induction of c-jun and c-fos in MC3T3-E1 cells, TPA induction of c-jun in cultured JB6 cells (Lu et al, 1994) and in NIH 3T3 cells (Lin et al, 1994) and TPA induced c-fos, c-jun and c-myc expression in mouse skin (Kakar and Roy, 1994) thereby inhibiting the transcriptional activators for AP-1. This has been confirmed since curcumin has been shown to inhibit the activation of c-Jun/AP-1 in mouse fibroblasts (Huang et al, 1991b), and in stromal cells (Xu et al, 1997). Curcumin can also block many reactions in which NF-κB plays a role (Chan, 1995; Singh and Aggarwal, 1995). It has been demonstrated to inhibit LPS induced activation of NF-κB in the macrophage cell line Mono-Mac-6 and in the human myelomonoblastic leukemia cell line, ML-1a (Singh and Aggarwal, 1995). Curcumin also inhibited TNFα and IL-1α induced NF-κB activation in stromal cells (Xu et al, 1997). All of these inhibitory effects may result in the suppression of tumour promotion, indicating that curcumin is probably a potent anti-tumour agent for some cancer cells. Zhang et al (1999) reported that curcumin inhibited COX-2 transcription in bile acid and phorbol ester treated gastrointestinal cells and directly inhibited the activity of COX-2. The pharmacological safety of curcumin is demonstrated by its consumption for centuries, at levels up to 100mg/day by people in certain countries (Ammon and Wahl, 1991). Therefore, curcumin, found in the human diet without demonstrating excessive toxicity, appears to have chemopreventive activity, that renders it worthy of further evaluation.

1.4.2.2: Caffeic acid phenethyl ester

CAPE, found in the propolis of honey bee hives, is structurally related to curcumin (Huang et al, 1996). The propolis of honey bee hives and honey has long been used in folk medicine as an anti-inflammatory agent with suspected anti-cancer activity (Huang et al, 1996). Although the propolis of honey bee hives is a complex mixture of compounds, including benzoic acid and their esters, substituted phenolic acids and
esters, flavanoid glycones and beeswax, caffeic acid 3,4 ester derivatives are the prominent chemical constituents that account for 20-25% of the total. Figure 1.4B shows the structure of CAPE.

CAPE is known to have anti-mitogenic, anti-carcinogenic, anti-inflammatory, anti-viral and immunomodulatory properties (Rao et al, 1993b). A study by Gribel and Pashinskii (1990) showed that honey possesses moderate anti-tumour and pronounced anti-metastatic effects in tumours from five different strains of rats and mice, and potentiated the anti-tumour effects of 5-fluoro-uracil and cyclophosphamide. Further evidence was demonstrated by Rao et al (1992) who showed that dietary CAPE and some other related compounds, inhibited colon adenocarcinoma HT-29 and HCT-116 cell growth, and exhibited differential toxicity to cancer cells versus normal cells. In addition these agents inhibited the AOM-induced colonic mucosal ODC, tyrosine protein kinase and COX activities, and colonic ACF in F344 rats (Rao et al, 1993b). Application of CAPE to mouse skin inhibited TPA-induced inflammation, ODC activity and formation of oxidised bases in epidermal DNA (Frankel et al, 1993). CAPE also suppressed TPA-mediated induction of COX-2 in human oral epithelial cells (Michaluant et al, 1999). These observations all suggest that CAPE may be an inhibitor of TPA induced tumour promotion.

Like curcumin, the mechanism by which CAPE inhibits colon tumourigenesis has not been established (Su et al, 1994). CAPE could suppress oxidative processes, for example CAPE reduced the levels of both intracellular H$_2$O$_2$ and oxidised bases in DNA of TPA treated human cells (Frankel et al, 1993; Huang et al, 1996). CAPE is a strong inhibitor of lipoxygenase and xanthine/xanthine oxidase activity in vitro (Sud'ina et al, 1993). CAPE has been shown to suppress lipid peroxidation (Laranjinha et al, 1995) and this is consistent with the antioxidant properties assigned to CAPE. However because of its diphenol structure CAPE may both scavenge free radicals and generate them by redox cycling under specific conditions. Chiao et al (1995) indicated that CAPE can act via a redox mechanism, as a prooxidant since they found that CAPE induced apoptosis was inhabitable by treatment with the antioxidant N-acetyl-cysteine (NAC). Alternatively CAPE could affect protein tyrosine kinase activity (Rao et al, 1993b), or modulate arachidonic acid metabolism. The anti-inflammatory properties of
CAPE have been attributed to suppression of eicosanoid synthesis (Mirzoeva and Calder, 1996).

CAPE exhibits differential toxicity to cancer cells compared to normal cells (Chiao et al, 1995). For example, CAPE has been shown to enhance apoptosis in cultured adenovirus-transformed rat embryo fibroblasts, compared to non-tumourigenic diploid parental cells. In addition, CAPE selectively inhibits the proliferation of several tumour and viral transformed rodent and human cells in culture, but not normal, non-tumourigenic cells (Chiao et al, 1995). A dose dependent inhibition of growth and DNA, RNA and protein synthesis was observed in HT-29 cells treated with CAPE (Su et al, 1994). Although the mechanisms for the multiple activities assigned to CAPE have not been defined, most of the beneficial properties of CAPE involve inhibition of NF-κB activity (Natarajan et al, 1996). CAPE has recently been shown to inhibit transcriptional activation by NF-κB at the level of DNA binding, and reducing agents such as dithiothreitol (DTT), reversed this effect, suggesting that CAPE may modify critical sulphhydryl groups (Natarajan et al, 1996). Natarajan et al (1996) found that CAPE can specifically inhibit NF-κB activation by diverse inflammatory agents, suggesting it can down-regulate NF-κB dependent gene expression. The protein kinases involved in signal transduction pathways where CAPE may have inhibitory activity are not known, but Jaiswal et al (1997) suggested that CAPE does not affect PKC activity.

1.4.2.3: Resveratrol

Resveratrol is a phytoalexin found in grapes, mulberries, peanuts and other food products. Fresh grape skin contains about 50-100μg of resveratrol/gram and the concentration in red wine is in the range of 1.5-3mg/litre. The structure of resveratrol, like that of CAPE bears some resemblance to curcumin, and is shown in Figure 1.4C. Resveratrol has been an important constituent of Japanese and Chinese folk medicine (Soleas et al, 1997). A large number of recent studies in cellular and animal models, demonstrated that resveratrol regulates many biological activities. Indirect evidence suggests that the presence of resveratrol in wine may explain the reduced risk of coronary heart disease associated with moderate wine consumption (Gaziano et al, 1993). This effect has been attributed to the inhibition of platelet aggregation, in
addition to the antioxidant and anti-inflammatory activities of resveratrol (Franzel et al, 1993).

Resveratrol has been shown to inhibit the development of preneoplastic lesions in carcinogen treated mouse mammary glands in culture and inhibited tumourigenesis in a two stage model of mouse skin cancer, that was promoted by treatment with phorbol esters (Jang et al, 1997). It has also been shown to inhibit the replication in vitro of human leukemia cells (Jang et al, 1997). In addition to these findings, increasing interest has been paid to resveratrol, since it has been shown to be an effective chemopreventive agent in assays representing the three major stages of carcinogenesis, initiation, promotion and progression (Jang et al, 1997). One tumour initiation event that resveratrol inhibited in a dose dependent way, was free radical formation after treatment of HL-60 cells with TPA (Sharma et al, 1994). The anti-mutagenic activity of resveratrol was shown by its inhibition of the mutagenic response induced by the carcinogens, 3-amino-1,4-dimethyl-5H-pyrido[4,3-β] indole and DMBA, in the Ames assay using Salmonella typhimurium. Resveratrol can also induce phase II drug metabolising enzymes (Jang et al, 1997).

Jang and Pezzuto (1998) suggested that the inhibitory effects of resveratrol on tumour promotion may be due at least in part to interference with ROS generating pathways and modulation of antioxidant defence systems. Jang et al (1997) reported that resveratrol can suppress prostaglandin synthesis via direct selective inhibition of the cyclooxygenase component of COX-1, not COX-2, and this activity correlated with its anti-tumour promotion effects. However more recently resveratrol has been shown to suppress TPA mediated induction of prostaglandin synthesis by inhibiting COX-2 gene expression and the enzyme activity of COX-2 (Subbaramaiah et al, 1998). Kawada et al (1998) have suggested that the major target of resveratrol could be the receptor tyrosine kinase-MAP kinase pathway in stellate cells or the inhibition of PLC activation. Ragione et al (1998) demonstrated that resveratrol inhibited HL60 cell proliferation by blocking the S to G2/M transition in the cell cycle. Whereas the ability of resveratrol to inhibit cellular events associated with tumourigenesis has been attributed to the anti-cyclooxygenase activity of resveratrol, the precise molecular mechanisms of its anti-tumour activity are not well understood.
1.5: NF-κB and tumour promotion

1.5.1: NF-κB as a transcriptional activator

The inducible eukaryotic protein transcription factor NF-κB is a member of the Rel family of proteins (Baeuerle and Baltimore, 1996) and like AP-1 is implicated in tumour promotion. NF-κB was first identified by Sen and Baltimore (1986) and functions to enhance the transcription of a variety of genes. It was originally found in B cells, as a transcriptional activator of the immunoglobulin Kappa light chain gene (Sen and Baltimore, 1986). NF-κB is responsible for the transcriptional activation of many genes, including a variety of cellular and viral genes (Leonardo and Baltimore, 1989; Baeuerle and Henkel, 1994). It is involved in the regulation of many genes which code for mediators of the immune (Baeuerle and Baltimore, 1996), acute phase and inflammatory responses (Baeuerle and Baltimore, 1991; Baeuerle and Henkel, 1994), such as in the induction of genes for the pro-inflammatory cytokines, such as TNFα and many other immunoregulatory genes, including interleukin-2 (IL-2) (Leung and Nabel, 1988), interleukin-3 (IL-3), IL-6 and class 1 and 11 major histocompatibility complexes. It is also a critical regulator of genes that function in cell proliferation and apoptosis, and the cell adhesion molecules, endothelial leukocyte adhesion molecule 1 (ELAM1), intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), (Iademarco et al, 1992). NF-κB activation may therefore be viewed as a key event in the signalling pathway leading to the change in expression of such genes (Brennan and O’Neill, 1998). Because these gene products are also known to play a major role in the pathogenic processes of various diseases, NF-κB has been implicated in many pathological conditions including AIDS, septic shock and asthma (Baeuerle and Henkel, 1994). Asahara et al (1995) have shown that NF-κB is activated in the synovium of patients with rheumatoid arthritis compared to patients with osteoarthritis. NF-κB genes have been implicated in tumourigenesis, particularly in the pathogenesis of lymphoid malignancies, for example the c-Rel gene has been found to be rearranged in some cases of lymphoid neoplasia (Lu et al, 1991). For these reasons NF-κB is regarded as a good therapeutic target. A common feature of genes possessing NF-κB binding sites allows them to be well suited for the rapid synthesis of protective and signalling proteins after exposure to a wide variety of stresses, since NF-κB
activation of gene transcription does not require \textit{de novo} protein synthesis (Lenardo et al, 1989; Koong et al, 1994). The DNA binding protein complex recognises a discrete nucleotide sequence (5'-GGGRNYYYCC-3'), (where R is an unspecified purine, Y is an unspecified pyrimidine, and N is any nucleotide), called the κB site, in the upstream regions of a variety of responsive genes (Blackwell and Christman, 1997).

1.5.2: Activators of NF-κB

Since NF-κB is a central mediator of the immune response, it is activated by a wide variety of extracellular stimuli, including bacterial LPS, pro-inflammatory cytokines, eg. interleukin 1β (IL-1β), TNFα (Beg and Baldwin, 1994), mitogens, various viral proteins, including human T-cell leukemia virus type 1 (HIV-1), physical stress, such as UV and ionising radiation, oxidants such as H₂O₂, a variety of chemical agents, such as phorbol esters and certain phosphatase inhibitors, such as okadaic acid (Siebenlist et al, 1994; Thanos and Maniatis, 1995; Verma et al, 1995; Baueerle and Baltimore, 1996). NF-κB is an oxidant sensitive transcription factor (Scoonbroodt et al, 1997), and most of the inducers of NF-κB seem to rely on the production of ROS (Schreck et al, 1991; Meyer et al, 1993; Boland et al, 1997). Three lines of evidence support the idea that ROS play a role in NF-κB activation. Firstly ROS added directly to the cells, in the form of H₂O₂ potently activates NF-κB in some cell lines (Staal et al, 1990; Schreck et al, 1991). Secondly agents that activate NF-κB in cells (including TNFα, IL-1β and ionizing radiation) produce oxidative stress (Schreck et al, 1992a). The third line of evidence for a link between ROS and NF-κB is supported by the fact that antioxidants, especially thiol containing compounds, such as NAC (Schreck et al, 1991) and dithiocarbamates (Schreck et al, 1992b) can inhibit NF-κB activation. The overexpression of antioxidative enzymes such as catalase (Schmidt et al, 1995), thioredoxin or glutathione peroxidase inhibit the induction of NF-κB, by preventing phosphorylation and subsequent degradation of IkB (Henkel et al, 1993). Mirochnitchenko and Inouye (1996) have shown that macrophages from transgenic mice that overexpress CuZn superoxide dismutase have decreased NF-κB activation in response to TPA compared to control mice. The ability of these and other antioxidants to block the activation of NF-κB and the phosphorylation of IkBα suggests that the redox state of the cell affects a common final pathway leading to NF-κB activation, and
that an oxidizing environment is required for transmission of the signal. At least two
models are possible: firstly an excess of reducing agent interferes with the signalling
apparatus, possibly by altering disulphide bonds or inhibiting lipid peroxidation and
secondly, oxygen radicals themselves are second messengers that directly mediate NF-
κB activation by all stimuli. One study has demonstrated that over-expression of
enzymes which affect the level of oxygen radicals in cells modulate NF-κB activation
(Schmidt et al, 1996). Apart from the oxidative activation of NF-κB, other studies have
somewhat paradoxically demonstrated that DNA binding by NF-κB can be inhibited by
the modification of a sensitive thiol at cysteine 62 in the p50 subunit (Matthews et al,
1992) and the thiol must therefore be maintained in a reduced state for DNA binding to
occur. It is hypothesised that oxidative inhibition of NF-κB may occur in the cytosol
and that NF-κB may be translocated to the nucleus as a reversibly oxidised protein
(Galter et al, 1994)

1.5.3: NF-κB family members

NF-κB complexes bind DNA as dimers constituted from a family of proteins designated
as the Rel/NF-κB family (Verma et al, 1995). The subunits belonging to the NF-κB
family comprise of five members in mammals, p50 (NF-κB1), p65 (Rel A), p52 (NF-
κB2), Rel B and c-Rel (Siebenlist et al, 1994). The Rel protein family has been divided
into two groups based on differences in their structures, functions and modes of
synthesis (Baeuerle and Henkel, 1994; Siebenlist et al, 1994). The first group comprises
of p50 and p52 which are synthesised as precursor proteins of 105 and 100 kDa
respectively. The second group of Rel proteins, which includes p65, c-Rel and Rel B are
not synthesised as precursors (Thanos and Maniatis, 1995). The NF-κB subunits are
related to one another through an N-terminal stretch of 300 amino acids called the Rel
homology domain (Grimm and Baeuerle, 1993; Beauerle and Henkel, 1994). This
region mediates DNA binding, protein dimerisation, nuclear localisation and binding of
the inhibitory protein IκB (Baeuerle and Henkel, 1994). NF-κB proteins are capable of
forming numerous homodimers and heterodimers with other family members which
interact with a series of similar DNA target sites, collectively called NF-κB sites, but
with different affinities (Kunsch et al, 1992). The dimeric composition of the NF-κB
have shown that different Rel protein dimers have different abilities to stimulate transcription even when bound to the same NF-κB motif. Since each combination of Rel proteins preferentially binds to a particular subset of κB-binding site sequences, this gives rise to selective transcriptional activation or attenuation (the latter of which has been observed with the non-transactivating p50 homodimeric form) (Lee et al., 1995), and different Rel dimeric complexes may serve to activate specific sets of genes (Roshak et al., 1996; Boland et al., 1997). While there are many different forms of NF-κB, the predominant form of NF-κB in unstimulated cells occurs in the cytoplasm as a heterodimer of two proteins p50 and p65 (Koong et al., 1994), and is a transcriptional activator whereas p50/p50 or p52/p52 homodimers generally repress transcription (Verma et al., 1995). However the constituents of the activated NF-κB may be different from cell to cell and vary with the nature of the stimulus. Different κB sites among gene promoters are bound with different affinities by the various dimers which therefore bind certain target genes preferentially (Muller et al., 1996).

1.5.4: Signalling pathways involved in the activation of NF-κB

The activity of NF-κB is regulated by its intracellular location. In resting, non-stimulated cells, the NF-κB dimer is sequestered in the cytosol, as an inactive complex (Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990), as a result of interaction with a group of inhibitory proteins collectively known as IκB (IκB-α, IκB-β, IκB-γ and bcl-3) (Baeuerle and Baltimore, 1988; Beg and Baldwin, 1993; Nolan et al., 1993). IκB-γ and bcl-3 are specific for NF-κB p50 dimers, and only IκB-α and IκB-β interact with p65 and c-Rel, indicating that the responsibility for regulating NF-κB activity is carried out by these IκB isoforms (Thompson et al., 1995). Whiteside et al. (1997) have recently isolated a novel IκB family member IκB epsilon (IκB-ε) which is found complexed with Rel A and c-Rel in resting cells. Therefore it could be speculated that different IκB molecules may control the regulation of different genes, binding to and inhibiting different populations of NF-κB molecules in the cytoplasm.

IκB proteins mask the nuclear localisation signal of NF-κB, thereby preventing NF-κB nuclear localisation (Beg et al., 1992). Stimuli, through specific activated protein kinases, cause phosphorylation of IκB which induces dissociation of NF-κB from IκB.
This allows exposure of a nuclear localisation signal on both the p50 and p65 subunits, and allows translocation of NF-κB to the nucleus (Thanos and Maniatis, 1995). NF-κB must translocate to the nucleus in order to exhibit specific DNA binding and regulate the transcription of specific genes. Constitutive NF-κB activity in the cell nucleus can only be detected in certain neurons (Kaltschmidt et al, 1994), thymocytes (Sen et al, 1995), some cells of the monocyte/macrophage lineage, mature B cells (Frankenberger et al, 1994; Conti et al, 1997) and breast tumour cells.

The signal transduction pathways linking receptor stimulation to NF-κB activation are poorly defined. Since a diverse number of stimuli activate NF-κB it is possible that there exists several signal transduction pathways, rather than a common one, and the convergence point may be the degradation of IκB bound to NF-κB (Naumann and Scheidereit, 1994). Phosphorylation at two specific residues, serine 32 and 36, on IκB-α (Brown et al, 1995; Traenckner et al, 1995), although not sufficient for its dissociation are thought to target its rapid ubiquitination on lysine 20 or 21 (Scherer et al, 1995; Baldi et al, 1996). Subsequent degradation by the 26S proteosome complex (Traenckner et al, 1994; Chen et al, 1995), facilitates NF-κB release and translocation into the nucleus to bind to its DNA sequence and induce transcription (Scherer et al, 1995). When serine residues at the N-terminus of IκB-α are mutated to alanine, signal induced degradation is prevented (Brown et al, 1995). The corresponding Ser19 and Ser 23 of IκB-β represent critical phosphorylated residues (Verma et al, 1995). This therefore shows that NF-κB is a cellular response that links cell surface receptor activation to transcriptional events in the nucleus (Heller and Kronke, 1994). However, independently of its rapid breakdown induced by several stimuli and compared with NF-κB protein subunits, IκB-α is inherently unstable and undergoes a continuous turnover. Functional NF-κB binding elements are present in the IκB-α genes (Le Bail et al, 1993; Ito et al, 1994). Degradation of IκB and release of NF-κB leads to transactivation of the IκB-α gene itself and rapid resynthesis of the IκB protein (Blackwell and Christman, 1997). This establishes an autoregulatory loop (Shao-Cong et al, 1993) in which newly synthesised IκB-α is thought to restore the cytoplasmic pool of NF-κB (Sun et al, 1993) resulting in transcription termination (Zabel and Baeuerle, 1990). This phenomenon does not require the prior phosphorylation or ubiquitination of IκB but still appears to be mediated by the proteasome. Degradation of IκB-α occurs
preferentially with all of the NF-κB inducers tested, while IκB-β responds to only a subset. For example treatment of cells with TNFα or TPA causes a transient activation of NF-κB and the transient loss of IκB-α. The activation of NF-κB is transient because NF-κB increases the expression of IκB-α which switches NF-κB from the nucleus back to the cytoplasm. In contrast induction with IL-1 or LPS results in degradation of both IκB-α and IκB-β and the transcriptional activity of NF-κB lasts for several hours following stimulation despite the presence of newly synthesised IκB-α (Thompson et al, 1995).

Signalling pathways that precede the dissociation of IκB which is intrinsic to NF-κB activity are beginning to be defined. It is recognised that the key step in the activation of NF-κB is the N-terminal phosphorylation of IκB, which is necessary for its ubiquitination and degradation, and is likely to be the main point of control through which diverse stimuli activate NF-κB (Verma et al, 1995). Much of the effort to understand the regulation of this signalling pathway, has focused on the identification of the protein kinase(s) responsible for this event (Stancovski and Baltimore, 1997). A number of kinases have been implicated in the phosphorylation of IκB (Schulze-Osthoff et al, 1997). There is evidence for the possible involvement of the Ras/Raf1-MAP kinase pathway (Finco and Baldwin, 1993), a sphingomyelinase-ceramide pathway, and PKA and PKC pathways (Ghosh and Baltimore, 1990), both classical PKCs and the calcium and diacylglycerol (DAG)-independent PKC zeta, causing phosphorylation of either tyrosine, serine or threonine residues (Steffan et al, 1995). The ability of phorbol esters to induce nuclear translocation of NF-κB led to the suggestion early on that classical PKCs might be a common upstream component in the activation pathway. Purified PKC was shown to phosphorylate IκB-α and release NF-κB in vitro (Ghosh and Baltimore, 1990). However other activators of NF-κB, such as TNFα can activate NF-κB independently of PKC and ceramide-dependent kinase activation. This suggested that PKC is not likely to be the only kinase involved in the signalling pathway resulting in the phosphorylation and degradation of IκB.

None of the above kinase signalling pathways have been shown to phosphorylate both serine 32 and 36 of IκBα. The most notable identification of an IκB-α kinase is a recently identified multi-subunit protein kinase. DiDonato et al (1997) have described
the purification and molecular cloning of a 900 kDa protein kinase complex, called the IkB kinase (IKK). IKK phosphorylates IkB-α and IkB-β at the sites serine 32/36 and serine 19/23 respectively, that mediate their ubiquitation and degradation (DiDonato et al, 1997). Evidence, from antisense deprivation and dominant negative mutation studies, indicates that IKK is the critical protein kinase mediating NF-κB activation by TNFα, IL-1α or phorbol esters (DiDonato et al, 1997; Zandi et al, 1997). IKK was originally cloned as a serine threonine kinase called conserved helix loop helix ubiquitous kinase (CHUK) (Connelly and Marcu, 1995).

One component of the IKK complex is IKKα (DiDonato et al, 1997) an 85kD polypeptide that interacts with another protein kinase involved in NF-κB activation called NF-κB inducing kinase (NIK) (Regnier et al, 1997). NIK is a member of the mitogen activated protein kinase kinase kinase (MAP3Ks) family (Regnier et al, 1997), and was originally isolated through its interaction with TNF-receptor associated factor (TRAF2), a member of a family of proteins that interact with the TNFα receptor (Malinin et al, 1997), and is a component of the TNFα signalling complex, which plays a critical role in NF-κB activation. Overexpression of NIK was able to induce NF-κB activation and kinase inactive NIK mutants blocked TNFα and IL-1 induced NF-κB activation. It is suggested that NIK itself does not phosphorylate IkB, but directly or indirectly interacts with IKKα, leading to phosphorylation of IkB-α on both of its N-terminal regulatory serines at residues 32 and 36 (Regnier et al, 1997). A second component of the IKK complex was identified by Woronicz et al (1997) called IKKβ an 87kDa protein that shares 52% homology and overall similarity with IKKα. It is thought that IKKα and IKKβ are both necessary for IkB phosphorylation and NF-κB activation, and both appear to make equal contributions to IkB kinase activity (Zandi et al, 1997). IKK enzymes (α and β) catalyse the transfer of phosphate moieties from ATP to IkB (Israel, 1997). If all stimuli known to activate NF-κB lead to the activation of IKKα, this could be the target for agents aimed at controlling the inflammatory response. MAP kinases have also been shown to affect these sites, either directly or indirectly, such as ERK kinase kinase 1 (MEKK1), an upstream kinase of JNK, found to be required for NF-κB activation by TNFα by directly activating the IkBα kinase complex (Lee et al, 1997). MEKK1 is related to NIK, and it is implicated in the
Figure 1.5: Proposed model for NF-κB activation in response to TNFα or oxidants. Multiple signaling pathways are activated in response to TNFα or reactive oxygen species (ROS) exposure that cumulate in NF-κB activation. NF-κB activation in response to TNFα requires the recruitment of the adaptor molecule TRAF2 to the intracellular domain of the TNF receptor. TRAF2 directly interacts with NIK. Subsequent steps involve the activation of a multisubunit IκB kinase (IKK). IκB phosphorylation results in its proteolytic degradation, and allows NF-κB to translocate to the nucleus and bind to DNA regulatory regions within NF-κB target genes.
pathway to activate JNK, because kinase inactive MEKK1 mutants block JNK activation by TNFα, but MEKK itself does not bind to TRAF2. It is still not clear whether all of the diverse stimuli known to activate NF-κB also lead to the activation of IKKα or IKKβ. Coimmunoprecipitation assays demonstrated an interaction between JNK and the c-Rel subunit of NF-κB (Meyer et al, 1996). A recent report suggested that the mitogen activated p90kDa ribosomal S6 protein kinase, implicated in NF-κB activation by stimuli such as phorbol esters (Schouten et al, 1997) functions as an IκB kinase. The p38 MAPK appear to be implicated in the activation of NF-κB regulated genes in response to cytokine exposure (Beyaert et al, 1996). IκB phosphorylation may be a site of integration of different signalling pathways (Lee et al, 1997; Schouten et al, 1997). However some differences to the signalling pathways above, have been noted in studies with pervanadate, a model of oxidative stress, where tyrosine phosphorylation of IκB also results in its dissociation, independently of proteosome mediated degradation (Imbert et al, 1996). Figure 1.5, shows possible signal transduction pathways that may be important for NF-κB activation.

1.5.5: NF-κB binding sites in the promoter region of the COX-2 gene

It has been suggested that overexpression of COX-2 in colon tumour cells is due to changes in transcriptional control. The isolation of genomic clones for COX-2 from different species, and characterisation of their promoter regions, have yielded insights into the regulation of this gene (Kutchera et al, 1996). The 5'-flanking regulatory region for the COX-2 gene has recently been isolated, and sequence analysis has identified a number of possible transcription factor binding motifs, which could be involved in the transcriptional control of COX-2 (Yang et al, 1997). The promoter region of COX-2 is shown in Figure 1.6. The 5' regulatory sequence contains consensus sequences for SP-1, NF-κB, NF-IL6, AP-1 and AP-2 sites, cyclic AMP response element (CRE), as well as a serum response element (Xie et al, 1993). The CRE has been shown to be necessary for COX-2 induction in phorbol ester differentiated U937 cells (Inoue et al, 1994), and the importance of both the CRE and NF-IL6 sites for COX-2 expression in LPS and phorbol ester-stimulated human vascular smooth muscle cells has been described (Inoue et al, 1995). In a murine osteoblast cell line NF-κB and NF-IL6 were important in
Figure 1.6: Schematic representation of the human COX-2 promoter. The potential response elements are shown based on sequence similarities to consensus response elements. Distances are given as nucleotide positions relative to the transcriptional start site at +1

From Inuoe et al, 1995
TNFα stimulated PGE2 production (Yamamoto et al, 1995). Various hormone response elements such as the glucocorticoid response element (GRE) and insulin were located in the 5' region, indicating that COX-2 may be regulated by these hormones (Yang et al, 1997).

There are two NF-κB consensus sites in the promoter region of the human COX-2 gene (Appleby et al, 1994), which flank an SP1 site. Tandem NF-κB sites have been shown to act synergistically in the transcriptional induction of a gene that controls HIV replication. First is the NF-κB 5’ site (5’GGGCCGGAGAAGGGGATTCCCTGCGCCCCC-3’), the underlined region within -455 to -428 bases from the transcriptional start site. The second is the NF-κB 3’ site; (5’-CAGGAGAGTGCGACTGCCCTTCTGCT-3’) element, underlined within -232 to 205 bases from the transcriptional start site (Schmedtje et al, 1997). NF-κB-5’ has been shown to have a role in the mechanism of COX-2 induction by TNFα in a murine osteoblast cell line (Yamamoto et al, 1995). More recently, Jobin et al (1998) reported a role of NF-κB in TNFα inducible COX-2 gene expression in HT-29 cells. NF-κB-3’ may play a role in facilitating the induction of COX-2 by LPS stimulated RAW 264.7 cells (Hwang et al, 1997) and phorbol ester. Schmedtje et al (1997) discovered that the NF-κB-3’ site is necessary for hypoxia-mediated COX-2 transcription in HUVEC cells. Signalling via the NF-κB pathway is involved in regulation of COX-2 expression induced by IL-1β in rheumatoid arthritis synoviocytes (Crofford et al, 1997) and in A549, pulmonary cells (Newton et al, 1997). Activation of NF-κB could be involved in COX-2 overexpression in response to tumour promoters such as FP-12 and TNFα.

1.5.6: Role of NF-κB in the inhibition or promotion of apoptosis

Many recent reports have implicated the Rel/NF-κB family of transcription factors in the promotion or the inhibition of apoptosis and therefore NF-κB might function as a regulatory checkpoint between apoptosis, proliferation and transformation (Zong et al, 1997; van Antwerp et al, 1998). However it is important to realise that the actual role of NF-κB factors in the apoptosis pathway may be quite complex, and that their ability to accelerate or attenuate apoptosis may vary in different cell types or in response to different death-inducing stimuli (Lipton, 1997). The anti-apoptotic function of NF-κB is
largely substantiated by studies in which fibroblasts and macrophages derived from RelA−/− mice were found to be more sensitive to the cytotoxic effects of TNFα and died within 8 hours of exposure to the cytokine, whereas the RelA+/+ wild type controls survived the treatment (Beg and Baltimore, 1996). Replacement of p65 into RelA-deficient cells restored resistance to TNFα mediated apoptosis, indicating a potentially important role for NF-κB in regulating apoptosis. An anti-apoptotic function of NF-κB was suggested from the observation that these transgenic mice that lack the NF-κB p65/Rel A gene die embryonically from extensive apoptosis within the liver (Beg et al, 1995). Recently NF-κB was found to protect cells from apoptosis induced by TNFα and various genotoxic agents (Liu et al, 1996; Wang et al, 1996; Van Antwerp et al, 1996). Inhibition of constitutive NF-κB activity in murine B cell lymphomas induced cell death (Wu et al., 1996). Inhibition of NF-κB by enhancement of the transcription of the IκBα gene, provided a mechanism for B cell apoptosis by TGFβ. Krishnamoorthy et al (1999) reported that the presence of the RelA subunit in the nucleus is essential for protection of photoreceptor cells against apoptosis mediated by an oxidative mechanism. This suggests that the activation of NF-κB probably functions to transcriptionally up-regulate a gene or group of genes encoding proteins involved in the protection against cell killing, for example COX-2. However there is also evidence for apoptosis promoting properties of NF-κB. Studies have shown that c-Rel present in the NF-κB complex, may function in the activation of a set of death genes where its elevated expression was shown to coincide with the onset of apoptosis (Abbadie et al, 1993). There is also evidence of a pro-apoptotic effect for Rel A activity, for example it was shown that serum starvation of a human embryonic kidney cell line caused cell death accompanied by the activation of Rel A containing NF-κB (Grimm et al, 1996). Glutamate was found to induce NF-κB in neuronal cells and this appeared to cause cell death, and aspirin protected these cells from the glutamate induced NF-κB activation and subsequent cell death (Grilli et al, 1996).

1.5.7: Inhibition of NF-κB activation

The inappropriate regulation of NF-κB and its dependent genes has been associated with various pathological conditions including toxic/septic shock, graft versus host reaction, acute inflammatory conditions, acute phase response, viral replication,
radiation damage, atherosclerosis and cancer (Baeuerle and Henkel, 1994). This makes selective inhibition of NF-κB an important target for therapeutic intervention in some of these conditions. Delineation of the pathway leading to NF-κB activation by tumour promoters such as FP-12 and TNFα may have important implications, since it may be possible to find agents that can block steps along the activation pathway. Also NF-κB, being a key mediator of TNFα responses is an attractive target for therapeutic intervention against inflammation and inflammatory diseases such as rheumatoid arthritis (Beg & Baltimore, 1996). A number of compounds have been identified that can suppress NF-κB activation in vitro, including antioxidants, protease inhibitors and salicylates (Blackwell and Christman, 1997). Sodium salicylate (NaSal) and aspirin were recently shown to inhibit activation of NF-κB by TNFα and other agents, which suggests that this may be involved in the anti-inflammatory effects of these drugs. Both curcumin and CAPE have been shown to inhibit NFκB activation (Singh and Aggarwal, 1995; Natarajan et al, 1996). Curcumin prevented the translocation of the p65 subunit of NF-κB from the cytoplasm to the nucleus, whereas CAPE inhibition was affected at the level of binding of NF-κB to DNA. Curcumin can inhibit at a step in the signal transduction pathway of NF-κB activation that occurs upstream of IκB phosphorylation, but after the point at which various signals transduced by different stimuli converge. Curcumin therefore has great potential for modulating the expression of genes regulated by NF-κB.
1.6: Aims of this study

The overall rational of the work described in this thesis is to allow further insights into the mechanisms of cancer chemoprevention. The overall aim is therefore to contribute to the body of knowledge which may ultimately lead to the reduction in the number of individuals with colon cancer. Specifically the aims are as follows:

(1) To further investigate the mechanisms in which two model carcinogens, TNFα and FP-12 promote colon cancer, by investigating their effects at the level of early response gene transcription.

(2) To explain the mechanisms by which these agents alter the expression of COX-2, thought to be pivotal in colon carcinogenesis, by understanding the specific molecular events and signal transduction pathways, especially the role of the transcription factor NF-κB in COX-2 regulation.

(3) To find out how chemopreventive salicylates and alternative agents to NSAIDs, such as diet derived agents related to curcumin, interfere with this event, ie inhibit the effects of TNF-α and FP-12 on early response gene expression.

(4) To determine if the chemopreventive effect of agents such as curcumin, can by blocking the overexpression of COX-2, induce apoptosis in normal human colon epithelial cells.
CHAPTER 2: MATERIALS AND METHODS
MATERIALS

2.1: Source of Materials

The following chemicals and reagents were obtained from the sources indicated:

**Amersham International Plc., Little Chalfont, Bucks**

Molecular weight markers- rainbow coloured protein 14.3-200kD, anti-mouse and anti-rabbit horseradish peroxidase linked whole antibodies, enhanced chemilluminescence (ECL) Western blotting detection reagents, ECL hyperfilm, Hybond-ECL membrane, $[^{32}P]$ adenosine 5'-triphosphate (ATP).

**Boehringer Mannheim**

Random hexamers.

**Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts**

N, N, N', N'-tetramethylethylenediamine (TEMED), ammonium persulphate (AMPS), 30% acrylamide/bis solution (37.5:1), Bio-Rad protein assay dye reagent.

**Collagen Corp.**

Vitrogen 100.

**Gibco BRL Ltd, Glasgow, Scotland**

Polyclonal anti-PKC zeta antibody, Dulbeccos minimal essential medium (DMEM), foetal calf serum (FCS), Glutamax I, Ca$^{2+}$/Mg$^{2+}$-free phosphate buffered saline (PBS) (10x), trypsin/EDTA (10x), Trizol® reagent, Taq DNA polymerase, 100bp DNA ladder, M-MLV reverse transcriptase, proteinase K, bovine serum albumin (BSA), RNasin.

**KJP, Milton Keynes, Beds**

Polaroid type 55 film.

**Pharmacia Biotech., St Albans, Herts**

Nap-5 columns, deoxynucleotide triphosphate (dNTP) set 100mM solutions, RNase A.
Promega, Southampton
T4 polynucleotide kinase, AP-1 oligonucleotide, NF-κB oligonucleotide.

Santa Cruz Biotechnology Inc.
NF-κB-p65 rabbit polyclonal IgG supershift antibody, IκBα rabbit polyclonal IgG, COX-2 goat polyclonal IgG, c-Fos rabbit polyclonal IgG, anti-goat-IgG-HRP.

Tissue Culture Services, Botolph Claydon, Bucks
Monoclonal anti-PKC alpha antibody.

Sigma Chemical Co., Poole, Dorset
Curcumin, CAPE, resveratrol and all other chemicals and reagents.

2.2: Solutions and Buffers

2.2.1: Cell culture

Trypsin/Ethilenediaminetetraacetic acid (EDTA) solution (1x)
10 ml Trypsin/EDTA 10x solution
90 ml PBS
The solution was stored at 4°C

2.2.2: RNA extraction and RT-PCR

dNTP mix

<table>
<thead>
<tr>
<th>dNTP</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μl 100 mM dATP</td>
<td>10 mM</td>
</tr>
<tr>
<td>10 μl 100 mM dGTP</td>
<td>10 mM</td>
</tr>
<tr>
<td>10 μl 100 mM dCTP</td>
<td>10 mM</td>
</tr>
<tr>
<td>10 μl 100 mM dTTP</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

made up to 100 μl with water.
The solution was stored at -20°C.
Agarose Gel

To prepare a 1% gel, 1g electrophoresis grade agarose was dissolved in 100ml tris-borate EDTA (TBE) buffer by heating in a microwave. Once the agarose was completely dissolved the solution was allowed to cool to approximately 50°C and ethidium bromide added before pouring into the casting tray.

Gel Loading Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>50ml Glycerol</td>
<td>50% (v/v)</td>
</tr>
<tr>
<td>1ml sodium phosphate 1M, pH7.0</td>
<td>10mM</td>
</tr>
<tr>
<td>0.25g bromophenol blue</td>
<td>0.25% (w/v)</td>
</tr>
<tr>
<td>0.25g xylene cyanol</td>
<td>0.25% (w/v)</td>
</tr>
</tbody>
</table>

made up to 100ml with distilled water.

The buffer was stored at -20°C.

Diethyl pyrocarbonate (DEPC) Treated Water

To prepare RNase free water 0.5ml DEPC was added to a litre of ultrapure water. It was shaken, left overnight and autoclaved to degrade the DEPC.

TBE buffer (5x)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>54g Tris base</td>
<td>0.45M</td>
</tr>
<tr>
<td>27.5g boric acid</td>
<td>0.45M</td>
</tr>
<tr>
<td>20ml 0.5M EDTA (pH8.0)</td>
<td>0.01M</td>
</tr>
</tbody>
</table>

The chemicals were dissolved in water, the volume adjusted to 1 litre and the solution autoclaved. Working solutions (1x, 0.25x) were diluted in water.

2M Sodium Acetate (NaAc)

16.41g NaAc
to 100ml with pure water
The pH was adjusted to pH7.0.
2.2.3: Nuclear protein preparation and EMSAs

Buffer A

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>100μl HEPES 1M, pH7.8</td>
<td>10mM</td>
</tr>
<tr>
<td>50μl KCl 2M</td>
<td>10mM</td>
</tr>
<tr>
<td>20μl MgCl₂ 1M</td>
<td>2mM</td>
</tr>
<tr>
<td>10μl DTT 1M</td>
<td>1mM</td>
</tr>
<tr>
<td>2μl EDTA 0.5M</td>
<td>0.1mM</td>
</tr>
<tr>
<td>10μl PMSF 0.4M</td>
<td>0.4mM</td>
</tr>
<tr>
<td>4μl NaF 0.5M</td>
<td>0.2mM</td>
</tr>
<tr>
<td>20μl Na orthovanadate 100mM</td>
<td>0.2mM</td>
</tr>
<tr>
<td>600μl leupeptin 5mg/ml</td>
<td>0.3mg/ml</td>
</tr>
</tbody>
</table>

to 10ml with distilled water.

The buffer was prepared omitting phenylmethylsulfonylfluoride (PMSF), DTT and leupeptin and stored at 4°C. These were added prior to use.

Buffer B

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ml Nonidet P-40</td>
<td>10% (v/v)</td>
</tr>
</tbody>
</table>

to 100ml with distilled water.

The buffer was stored at 4°C.
**Buffer C**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>250μl HEPES 1M, pH 7.8</td>
<td>50mM</td>
</tr>
<tr>
<td>125μl KCl 2M</td>
<td>50mM</td>
</tr>
<tr>
<td>500μl NaCl 3M</td>
<td>300mM</td>
</tr>
<tr>
<td>1μl EDTA 0.5M</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>5μl DTT 1M</td>
<td>1mM</td>
</tr>
<tr>
<td>8μl PMSF 0.4M</td>
<td></td>
</tr>
<tr>
<td>500μl glycerol</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>2μl NaF 0.5M</td>
<td>0.2mM</td>
</tr>
<tr>
<td>10μl Naorthovanadate 0.1M</td>
<td>0.2mM</td>
</tr>
</tbody>
</table>

To 5ml with distilled water.

The buffer was prepared omitting PMSF and DDT and stored at 4°C. DDT and PMSF were added prior to use.

**Acrylamide gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>8ml acrylamide</td>
<td>4% (v/v)</td>
</tr>
<tr>
<td>3ml 5x TBE</td>
<td></td>
</tr>
<tr>
<td>0.4ml AMPS (10% solution)</td>
<td></td>
</tr>
<tr>
<td>100μl TEMED</td>
<td></td>
</tr>
<tr>
<td>49.25ml water.</td>
<td></td>
</tr>
</tbody>
</table>

The AMPS and TEMED were added just before pouring the gel.

**Loading buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>20ml glycerol</td>
<td>20% (v/v)</td>
</tr>
<tr>
<td>DTT</td>
<td>2mM</td>
</tr>
<tr>
<td>5ml 5x TBE</td>
<td>0.25x</td>
</tr>
<tr>
<td>0.1g bromophenol blue</td>
<td>0.1%</td>
</tr>
</tbody>
</table>
2.2.4: Nuclear, cytoplasmic and membrane protein extraction

Sonication Buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>121.1mg Tris base</td>
<td>10mM</td>
</tr>
<tr>
<td>584.4mg NaCl</td>
<td>100mM</td>
</tr>
<tr>
<td>40.7mg MgCl₂</td>
<td>2mM</td>
</tr>
<tr>
<td>190.2mg EGTA</td>
<td>5mM</td>
</tr>
<tr>
<td>314μl β-mercaptoethanol</td>
<td>45mM</td>
</tr>
<tr>
<td>1g Nonidet P-40</td>
<td>1% (w/v)</td>
</tr>
</tbody>
</table>

made up to 100ml with distilled water.
The pH was adjusted to 8.0 with NaOH and stored at 4°C. Leupeptin and aprotinin were added prior to use at final concentrations of 15μg/ml.

Swelling Buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>373.0mg KCl</td>
<td>10mM</td>
</tr>
<tr>
<td>1.82g Tris base</td>
<td>30mM</td>
</tr>
<tr>
<td>5.36mg MgAc</td>
<td>5mM</td>
</tr>
<tr>
<td>951.0mg EGTA</td>
<td>5mM</td>
</tr>
<tr>
<td>1.57ml β-mercaptoethanol</td>
<td>45mM</td>
</tr>
<tr>
<td>783.0mg benzamide</td>
<td>10mM</td>
</tr>
</tbody>
</table>

to 500ml with distilled water.
The pH was adjusted to 7.5 with HCl and stored at 4°C. Leupeptin and aprotinin were added prior to use to give final concentrations of 15μg/ml.

Swelling Buffer with 25% Glycerol

This was prepared as for swelling buffer, but 25ml distilled water was replaced with 25ml glycerol prior to pH adjustment. The protease inhibitors were added as for the swelling buffer.
Swelling Buffer, 25% Glycerol and 0.1% Triton X100
5ml swelling buffer with 25% glycerol
25μl Triton x100, 20% (w/v) in distilled water.
The protease inhibitors were added as for the swelling buffer.

Swelling Buffer and 1% Nonidet P-40
2ml Swelling buffer
20mg Nonidet P-40.

Wash Buffer

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.32g KCl</td>
<td>125mM</td>
</tr>
<tr>
<td>3.63g Tris base</td>
<td>30mM</td>
</tr>
<tr>
<td>1.07g MgAc</td>
<td>5mM</td>
</tr>
<tr>
<td>1.90g EGTA</td>
<td>5mM</td>
</tr>
<tr>
<td>3.14ml β-mercaptoethanol</td>
<td></td>
</tr>
</tbody>
</table>

made up to 1 litre with distilled water.
The pH was adjusted to 7.5 with HCl and stored at 4°C. Leupeptin and aprotinin were added prior to use to give final concentrations of 2μg/ml.
2.2.5: Whole cell protein extract

**H8 Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>200μl Tris-HCl 1.0M, pH 7.5</td>
<td>20mM</td>
</tr>
<tr>
<td>200μl EDTA 100mM, pH 7.5</td>
<td>2mM</td>
</tr>
<tr>
<td>200μl EGTA 100mM, pH 7.5</td>
<td>2mM</td>
</tr>
<tr>
<td>4μl β-mercaptoethanol</td>
<td>6mM</td>
</tr>
<tr>
<td>10μl leupeptin 2mg/ml</td>
<td>2μg/ml</td>
</tr>
<tr>
<td>10μl aprotinin 2mg/ml</td>
<td>2μg/ml</td>
</tr>
</tbody>
</table>

made up to 10ml with distilled water.

**W3 Wash Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.42g Tris base</td>
<td>20mM</td>
</tr>
<tr>
<td>8.77g NaCl</td>
<td>150mM</td>
</tr>
<tr>
<td>0.90g glucose</td>
<td>5mM</td>
</tr>
</tbody>
</table>

made up to 1 litre with water.

The pH was adjusted to 7.4 with concentrated HCl and stored at 4°C. Prior to use, protease inhibitors, leupeptin and aprotinin were added to give a final concentration of 2μg/ml.

2.2.6: Western blots

**Blotting Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.64g Tris base</td>
<td>48mM</td>
</tr>
<tr>
<td>5.84g glycine</td>
<td>39mM</td>
</tr>
<tr>
<td>0.76g sodium dodecyl sulphate (SDS)</td>
<td>0.0375% (w/v)</td>
</tr>
<tr>
<td>400ml methanol</td>
<td>20% (v/v)</td>
</tr>
</tbody>
</table>

made up to 2 litres with distilled water.

The solution was stored at 4°C.
**Electrode Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0g Tris base</td>
<td>25mM</td>
</tr>
<tr>
<td>14.5g glycine</td>
<td>192mM</td>
</tr>
<tr>
<td>1g SDS</td>
<td>0.1% (w/v)</td>
</tr>
</tbody>
</table>

made up to 1 litre with distilled water.
The buffer was stored at 4°C.

**Sample Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ml Tris-HCl 500mM, pH6.8</td>
<td>125mM</td>
</tr>
<tr>
<td>8ml glycerol</td>
<td>20% (v/v)</td>
</tr>
<tr>
<td>16ml SDS 10% (w/v)</td>
<td>4% (w/v)</td>
</tr>
<tr>
<td>2ml bromophenol blue (1mg/ml)</td>
<td>50μg/ml</td>
</tr>
<tr>
<td>1.23g DTT</td>
<td>200mM</td>
</tr>
</tbody>
</table>

made up to 40ml with distilled water.
The buffer was prepared omitting DTT and stored at room temperature. DTT was added prior to use.

**Separating gel for COX-2 analysis**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0ml Tris-HCl 0.75M, pH8.8</td>
<td>375mM</td>
</tr>
<tr>
<td>0.2ml SDS 10% (w/v)</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>6.7ml 30% acrylamide/bis (37.5:1)</td>
<td>10% (w/v)</td>
</tr>
<tr>
<td>3.34ml distilled water</td>
<td></td>
</tr>
<tr>
<td>200μl AMPS 10% (w/v)</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>7.5μl TEMED</td>
<td></td>
</tr>
</tbody>
</table>

The separating gel was prepared just before pouring. AMPS catalyses polymerisation and TEMED accelerates the reaction and so these two reagents were added last.

**Separating gel for IκB analysis**

This was prepared as for the COX-2 gel except a 12.5% acrylamide/bis final concentration was prepared. Thus 8.4ml acrylamide/bis with 1.2ml water was added.
Separating gel for PKC analysis
This was prepared as for the COX-2 gel except an 8% acrylamide/bis final concentration was prepared. Thus 5.3ml acrylamide/bis and 4.2ml water was used to prepare the gel.

Stacking gel (PKC and IkB)

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5ml Tris-HCl 0.5M, pH6.8</td>
<td>125mM</td>
</tr>
<tr>
<td>0.1ml SDS 10% (w/v)</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>1.6ml 30% acrylamide/bis</td>
<td>5% (w/v)</td>
</tr>
<tr>
<td>5.7ml distilled water</td>
<td></td>
</tr>
<tr>
<td>50μl AMPS 10% (w/v)</td>
<td>0.05% (w/v)</td>
</tr>
<tr>
<td>5μl TEMED</td>
<td></td>
</tr>
</tbody>
</table>

The stacking gel was prepared just before use.

Stacking Gel for COX-2
This was prepared as for the stacking gel above, except a 3.5% final concentration of acrylamide/bis was achieved.

Stain/Destain

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50ml Acetic acid</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>100ml Methanol</td>
<td>20% (v/v)</td>
</tr>
<tr>
<td>1.25g coomassie blue</td>
<td>0.25% (w/v)</td>
</tr>
</tbody>
</table>

made up to 500ml with distilled water.
The destain was made as above, for the stain, but with the omission of the coomassie blue. The solutions were stored at room temperature.

Stripping Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.78g Tris base</td>
<td>62.5mM</td>
</tr>
<tr>
<td>10g SDS</td>
<td>2% (w/v)</td>
</tr>
<tr>
<td>3.49ml β-mercaptoethanol</td>
<td>100mM</td>
</tr>
</tbody>
</table>

made up to 500ml with distilled water
The pH was adjusted to 6.7 and stored at room temperature.

**Tris buffered saline – Tween 20 (0.1%) (TBS-T20)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.42g Tris base</td>
<td>20mM</td>
</tr>
<tr>
<td>8g NaCl</td>
<td>137mM</td>
</tr>
<tr>
<td>1g T20</td>
<td>0.1% (w/v)</td>
</tr>
</tbody>
</table>

To 1 litre with distilled water.

The pH was adjusted to 7.6 with concentrated HCl and stored at 4°C.

### 2.2.7: DNA laddering gel

**Gel loading solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4µl 75% Ficoll 70</td>
<td>15% (w/v)</td>
</tr>
<tr>
<td>4µl 5x TBE</td>
<td>1x TBE</td>
</tr>
<tr>
<td>1µl 0.2% bromophenol blue</td>
<td>0.01% (w/v)</td>
</tr>
</tbody>
</table>

### 2.2.8: MTT assay

MTT (5mg/ml) was made up in calcium/magnesium free PBS just before use. The solution was filtered through a 2µm filter and the solution was stored at room temperature and covered with foil. The stock solution was diluted 1:5 with DMEM/2% FCS prior to use.

**METHODS**

### 2.3: Cell culture

#### 2.3.1: Routine cell maintenance

Cell culture was carried out aseptically in a class II laminar flow cabinet. Cells were maintained in a Sanyo CO₂ auto-zero incubator at 37°C in a humidified 95% air: 5% CO₂ atmosphere. Human colon epithelial cells (HCEC) were provided by Dr Andrea
Pfeifer (Nestec Ltd. Research Centre, Lausanne, Switzerland), and grown as a monolayer culture. These cells are normal human epithelial cells that have been immortalised by infection with the SV40 large T antigen. Cells were routinely passaged twice weekly, when they were confluent, to maintain logarithmic growth. Cells were used for experiments between passage numbers 3-20.

HCEC cells were grown in DMEM with 4500mg/l glucose, supplemented with Glutamax and 10% FCS. Before seeding and plating cells, tissue culture vessels were precoated with DMEM medium containing Vitrogen 100 (10μl/ml), human fibronectin (2.5μg/ml) and BSA (50μg/ml) (coating medium). The coating medium was added to the flasks after filter sterilisation (0.22μM) and incubated for 30 minutes at 37°C, and then any excess removed. Cells were briefly washed with 1X trypsin/EDTA solution, and then incubated for 5 minutes by addition of a further 5ml of 1X trypsin/EDTA, at 37°C, to detach adherent cells. Cell pellets were resuspended by the addition of 10ml prewarmed medium with several pipette mixings. The cells were counted and added at the desired density to precoated tissue culture vessels with medium.

SW480 colon carcinoma cells were a gift from Professor Christos Paraskeva (Bristol University, UK) and were cultured as for HCEC cells, but without precoating the flasks.

2.3.2: Cell storage in Liquid nitrogen

HCEC cells were frozen at low passage number to provide stocks of cells. Cells were trypsinised and pelleted using a Sorval TC6 centrifuge (Dupont) at 1000rpm for 10 minutes and resuspended in 90% FCS, 10% dimethylsulphoxide (DMSO) to a density of 2-3 X 10^6 cells/ml. Aliquots of 1ml were frozen at -80°C overnight and then immersed in liquid nitrogen. Cells were revived by rapid thawing to room temperature and each vial was transferred to a flask containing prewarmed culture medium.

2.3.3: Treatment of cells with FP-12 and TNFα

When the HCEC cells reached confluency, the DMEM medium containing 10% FCS was replaced with medium containing 2% FCS. The cells were incubated under these conditions for 24 hours to reduce any residual serum induced growth factors that may
be present in the culture medium. Treatment of cells with FP-12 and TNFα was performed on cultures containing approximately $1 \times 10^6$ cells/ml (approximate number when confluent), in 10ml culture medium, in 90cm$^2$ petri dishes.

The handling and storage of FP-12 was carried out according to previously described procedures (Plummer and Faux, 1994). FP-12 was purchased from SRI International (Menlo Park, USA) and dissolved in EtOH/DMSO (1:1 v/v) at 5mg/ml to provide a 20mM stock. FP-12 was aliquoted and stored in liquid nitrogen at -70°C. FP-12 was checked spectrometrically with a Unicam SP8-400 UV-Vis spectrophotometer (Pye Unicam, Cambridge, UK). Immediately prior to cell treatment FP-12 was diluted 1:5 with ethanol. TNFα was made as a 10μg/ml stock in PBS, aliquoted and stored at -20°C. Curcumin and CAPE were made as a 20mM stock in DMSO and stored at -20°C in light impervious tubes. Control cultures received equivalent dosing with solvent only.

2.4: Assays for estimation of cell viability

2.4.1: Trypan blue exclusion assay

In order to assess the physiological relevance of the doses of compounds used in these studies, the cytotoxic effects of the various agents was measured. Increased membrane permeability allows dye entry, for example of Trypan blue.

After treatment the cells were washed with Trypsin and incubated at 37°C for 5 minutes after the addition of a further 5ml, to detach the cells (section 2.3.1). After incubation 5ml tissue culture medium was added to stop the reaction. The cells were collected by centrifugation at 1000rpm for 5 minutes and resuspended in 30μl DMEM tissue culture medium. 10μl of the resuspended cells were mixed with 10μl Trypan blue. The number of cells stained with trypan blue and unstained cells were counted using a haemocytometer, under a phase contrast light microscope. Dead cells stain blue. At least 4 random fields were counted, and at least 500 cells/field.
2.4.2: MTT assay

The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is taken up into cells and reduced in a mitochondria-dependent reaction to yield a blue formazan product. The product accumulates within the cell, due to the fact that it can not pass through the plasma membrane. On solubilisation of the cells in organic solvent, the blue product is liberated and can be readily detected and quantified by a simple colorimetric method. The amount of formazan produced is directly proportional to the number of viable cells present. The assay has therefore been adapted for the measurement of cell survival and/or cell number.

A 96 well dish was precoated with 50μl coating medium (section 2.3.1) / well, for 30 minutes. HCEC cells were detached from a confluent flask (section 2.3.1), and counted with a haemocytometer. The cells were diluted to ~0.13 x 10^6 cells/ml with DMEM (10% FCS). Excess coating medium was removed from the wells and 150μl HCEC cells (approximately 2 x 10^4 cells) were pipetted into the 96 well microtitre plate with a multi-tip pipettor (gilson). The cells were left to attach overnight. Subsequently the cells were serum starved in DMEM (2% serum) overnight.

After serum starving the medium was removed from the wells. The cells were treated in triplicate in 150μl DMEM. After treatment with TNFα (with and without 1 hour pretreatment with curcumin or CAPE) for 24 hours, 150μl fresh DMEM/2% FCS was added to the wells and left overnight for the dead cells to detach. 50μl MTT solution (section 2.2.8) was added per well, and incubated for 2 hours at 37°C. After incubation, the MTT solution was removed completely from the wells and replaced with 100μl DMSO. The formazan crystals were dissolved by gentle shaking of the plate, prior to reading the absorbance at 540nm with a Labsystems plate reader.

Equation 2.1: Percentage of cell survival is expressed as:

\[
\text{(Absorbance of treated cells / absorbance of control cells) x 100%}
\]
2.5: Preparation of total RNA

Total RNA was prepared from cells using TRIZOL® reagent. Trizol reagent is a solution of phenol and guanidine isothiocyanate, and during sample lysis it maintains the integrity of the RNA, while disrupting cells and dissolving cell components.

Briefly 1.5ml Trizol was added to approximately 1X 10^7 cells in 90cm^3 dishes and the cell lysate was passed several times through a pipette. The homogenised samples were incubated at room temperature for 5 minutes to permit the complete dissociation of the nuclear protein complex. 0.3ml chloroform was added, shaken vigorously and left to stand at room temperature for 2-3 minutes, followed by centrifugation at 9500rpm for 15 minutes at 4°C. This separates the solution into an aqueous phase, containing RNA, an interphase containing DNA, and a lower red organic phase containing protein. After transfer of the aqueous phase containing RNA to a fresh tube, the RNA was recovered by precipitation with 0.75ml isopropanol. This was incubated at room temperature for 10 minutes followed by centrifugation for 5 minutes at 7500rpm at 4°C. The RNA pellet was washed once with ice cold 75% ethanol, in DEPC treated water (section 2.2.2) and centrifuged for 5 minutes, 7500rpm at 4°C. The RNA pellet was allowed to briefly air dry and was resuspended in 50μl DEPC water. The samples were stored at -80°C. The optical density at 260 and 280nm was measured using a Perkin Elmer λ2S spectrophotometer. The RNA concentration was then calculated using equation 2.2.

Equation 2.2: RNA mg/ml= OD 260nm x 40* x dilution factor
*(since for RNA an OD_{260} of 1 = 40μg/ml).

The OD ratio 260:280nm was measured to determine the purity of the RNA samples. Only RNA samples with a ratio greater than 1:1.5 were used in subsequent RT-PCR reactions.

2.6: Reverse Transcription Polymerase Chain Reaction

Reverse transcription of RNA followed by the polymerase chain reaction (RT-PCR) can be used to detect low amounts or even single copies of RNA. Reverse transcription transcribes RNA into complementary single stranded DNA (cDNA). PCR uses the cDNA as a template resulting in numerous copies of a specific sequence of DNA. Semi-
quantitative RT-PCR analysis of COX2 mRNA levels was performed according to the method of Hla and Maciag (1991), with some modifications. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and COX-2 are shown in Figure 2.1. Primers were synthesised to amplify the cDNA encoding human GAPDH, a constitutively expressed gene, to act as control.

cDNA was prepared by reverse transcription of 1μg of total RNA extracted from cells using Trizol® reagent (section 2.5). The RNA was reverse transcribed in a total volume of 10μl reverse transcriptase (RT) buffer, containing PCR buffer (1x), MgCl₂ (5mM), RNasin (1 unit/μl), maloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (10 units/μl), dNTPs (1mM of each dATP, dGTP, dTTP and dTTP) (section 2.2.2), random hexamers (15 pmoles/μl) and DDT (1mM). Mineral oil (2 drops~50μl) was laid on top of the reaction mixtures to prevent any evaporation. A Hybaid Omnigene PCR machine was used. Reaction mixtures were incubated at 23°C for 10 minutes, 42°C for 45 minutes and 99°C for 10 minutes.

After reverse transcription, the cDNA was diluted 1 in 10 by the addition of 90μl of distilled water. An aliquot (10μl) of this cDNA was used for amplification in the PCR reaction. PCR reactions were performed in 40μl PCR buffer, containing sense and antisense primers (1.25 pmoles/μl of each) for GAPDH or COX-2 (Figure 2.1), PCR buffer (1x), MgCl₂ (2.5mM), dNTP's (1mM) of each (section 2.2), Taq DNA polymerase (0.2 units/μl) and water (to make the volume up to 40μl). Mineral oil (~100μl) was laid on top of the reaction mixtures. The PCR reactions were incubated at 94°C for 3-4 minutes to denature the DNA/RNA hybrids, followed by 25 cycles of denaturing at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 1 minute. A final extension of 5 minutes at 72°C was carried out and the samples were stored at 4°C.
Figure 2.1: The oligonucleotide primers for human COX-2 and GAPDH

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense/Olive</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>Sense</td>
<td>5' TTCAAATGAGATTGTGGGAAAATTGCT-3'</td>
<td>573</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5' AGATCATCTCTGCCTGAGTATCTTT-3'</td>
<td>878</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>5'CCACCCATGGCAAATTCCATGGCA-3'</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'TCTAGACGGCAGGTCAGGTCCACC-5'</td>
<td>89</td>
</tr>
</tbody>
</table>

2.7: Gel Analysis of PCR Products

The COX-2 and GAPDH amplification products were separated by electrophoresis in a 1.5% agarose gel, containing ethidium bromide (3µl at 5µg/ml per 35ml gel), using 1X TBE as the running buffer (section 2.2.2). An aliquot (10µl) of the PCR product was mixed with 1µl gel loading buffer (section 2.2.2) and run at 80 volts for 35 minutes. The gel was visualised under a UV transilluminator, and photographed with Polaroid type 55 film. The Polaroid negative was soaked in an 18% sodium sulphite solution for 5 minutes, and then overnight, in water with detergent. Finally the negative was soaked again overnight in ultra-pure water. The Polaroid negative was used for densitometric band intensity measurements with a Molecular Dynamics computing densitometer using Image Quant software.

The intensity of the GAPDH band (a housekeeping gene) for each starting RNA sample was measured, in parallel to COX-2 to normalise between differences in starting amounts of samples. For each sample the integrated optical densities of the COX-2 band were divided by that of the GAPDH band to rectify any errors in quantification, that may have been caused by adding different amounts of RNA into each reaction. To ensure that the data obtained was quantitative (relative to control, GAPDH) both products, GAPDH and COX-2 were amplified for a number of cycles which ensured that the amount of amplified product was directly proportional to the input RNA; the number of cycles used in this assay was determined by finding the midpoint of the curve when the cycle number was plotted against band density.
2.8: Nuclear Protein Purification

Subsequent to appropriate treatment nuclear protein extracts were isolated from HCEC cells (2 X 10^7 cells total), according to the method of Staal et al (1990). All isolation steps were performed on ice. The cells were washed twice in ice cold calcium magnesium free PBS, and harvested by scraping into the residue. Cells were isolated by centrifugation, in a 1.5ml microfuge tube, at 1200rpm, for 10 minutes at 4°C. The cells were washed in 1ml PBS and pelleted at 14000rpm for 15 seconds at 4°C and resuspended in 0.4ml of ice cold cell lysis buffer (buffer A, section 2.2.3). After 15 minutes incubation on ice, 25μl of buffer B (section 2.2.3) was added. This was mixed vigorously by vortexing for 15 seconds, and centrifuged in a microcentrifuge for 30 seconds at 14000rpm at 4°C. This facilitated disruption of the plasma membrane and cell lysis whilst retaining the integrity of the nuclei. The pellet generated by this step contained purified nuclei. The supernatant was removed and the nuclear pellet was resuspended in 50μl of ice cold nuclear protein isolation buffer (buffer C, section 2.2.3), and gently mixed on a rotating platform at 4°C for 20 minutes. The nuclear proteins were then extracted by centrifugation at 14000rpm for 5 minutes at 4°C and the supernatant, designated as the nuclear protein was carefully decanted and stored at -80°C until required. The protein concentration was measured with the Bradford method (Bradford, 1976).

2.9: Electrophoretic gel mobility shift assay (EMSA).

The electrophoretic gel mobility shift assay relies on the ability of protein and DNA complexes to migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments. The assay can therefore be used to assess the activity of transcription factors in a nuclear lysate. EMSA is performed by incubating nuclear protein extracts with a 32P-end-labelled oligonucleotide containing the protein binding site, and its electrophoretic mobility assessed on a nondenaturing polyacrylamide gel.

A double stranded oligonucleotide containing the sequence corresponding to the consensus sequence of NF-κB ('5-AGTTGAGGGGACTTTCCCAGGC-3') (with the factor binding site underlined), from the κ light chain enhancer, was end labelled with [γ-32P] ATP using T4 polynucleotide kinase. In end labelling the 5' OH group of the
terminal nucleotide is replaced with a $\gamma^{32}$P from the $\gamma^{32}$P ATP. 5μl of the NF-κB oligonucleotide, 1μl T4 polynucleotide kinase, 2μl T4 polynucleotide kinase buffer, 5μl [$\gamma^{32}$P] ATP and 7μl water were incubated at 37°C for 30 minutes. 80μl TE buffer was added to stop the reaction. To purify the radiolabelled oligonucleotide, the sample was applied to a Nap5 column and the eluate collected. The oligonucleotide was further eluted in fractions through the Nap5 column by the addition of 100μl TE aliquots to the column to remove unincorporated nucleotides from the DNA probe. A further 11 fractions were collected in fresh eppendorf tubes. The fractions were counted for 1 minute using a bench count (Scotlab Easicount 2000) to obtain the disintergrations per minute (dpm) value for each fraction. The dpm counts were maximal in fractions 8-9 and then started to decrease. The peak in the number of counts represents the labelled oligonucleotide. The fractions (2-3 fractions) with the most radioactivity were combined and reprecipitated overnight at -20°C, with 1/10 volume 5M NaCl and 2.5 volumes of ethanol. The mixture was spun down at 13,500 rpm for 30 minutes and dissolved in 50μl water. The labelled oligonucleotide was added to the reaction mixture at ~ 0.25 pmoles final concentration.

Nuclear protein extract (4μg) was incubated for 30 minutes at room temperature in 20μl DNA binding buffer, containing 20mM Hepes (pH 7.5), 4% Ficoll, 0.5μg/ml poly dIdC, 0.1mM MgCl$_2$, 0.1mM DTT, and 1μl $^{32}$P-end-labelled double stranded oligonucleotide probe containing the consensus NF-κB site (or AP-1 site). Before loading the samples on the gel 2μl loading buffer (section 2.2.3) was added to each sample. The reaction mixtures (DNA/protein product formed) were separated from free oligonucleotide, in a precooled, non-denaturing 4% polyacrylamide gel (29:1, acrylamide:bisacrylamide) (section 2.2.3) and electrophoresed in 0.25 X TBE buffer, with buffer recirculation, for 10 minutes at 240 volts, and for a further 2 hours at 120 volts, or until the bromophenol blue dye was two thirds of the way down the gel. Following electrophoresis, the gel was dried under vacuum for 45 minutes at 80°C, and visualised by autoradiography by exposure to Kodak X-Omat film at -70°C with intensifying screens. The film was developed using an automatic developer (X-graph Compact X2). Subsequently the radioactivity in the retarded binding complexes was quantified, by exposing the dried gel in phosphorimager plates and measuring band intensities with a phosphorimager (Molecular Dynamics), Bucks, UK, using image-quant™ software.
To determine the specificity of the gel shift complexes (specificity of binding to the oligonucleotides) competition studies were performed. 200 fold molar excess of an unlabelled NF-κB oligonucleotide that competed away all binding complexes (cold NF-κB) or an unlabelled oligonucleotide containing the consensus AP-1 binding sequence (5'CGCTTGATGAGTCAGCCGGAA-3') were added, in the binding buffer prior to separation of the protein/DNA complexes in the polyacrylamide gel. The AP-1 oligonucleotide does not compete with NF-κB for binding, demonstrating specificity of NF-κB binding to the labelled oligonucleotide.

In parallel EMSAs, specific supershift antibodies against the p65 component of human NF-κB was added to the binding reaction. The nuclear protein extracts were incubated with 2μl of p65 antibody (2mg/ml) or an antibody to c-Fos, to act as a negative control, for 30 minutes at room temperature, in the binding conditions, without the 32P labelled oligonucleotide probe. After this incubation, the probe was added and incubated for a further 30 minutes, prior to polyacrylamide gel electrophoresis.

2.10: Reprecipitation of oligonucleotides

Complementary oligonucleotides with sequences derived from the human COX-2 promoter region with homology to consensus NF-κB binding sites were synthesised, by the protein and nucleic acid chemistry laboratory. A mutated version of this oligonucleotide was also synthesised to examine the specificity of binding of NF-κB to the COX-2 promoter. The sequences of these oligonucleotides are shown in Figure 2.2. The oligonucleotides were delivered in an ammonium solution and needed to be extracted by reprecipitation and annealing before use.
Figure 2.2: Nucleotide sequences of NF-κB oligonucleotides in the promoter region of the COX-2 gene and the corresponding mutated sequences

NF-κB2 f  5'GAGAGTGGGGACTACCCCCTCTC-3'
NF-κB2 r  5'GCAGAGGGGTAGTCCCACCTTC-3'
NF-κB2M f  5'GAGAGTGGA ACTACCCCTCTC-3'
NF-κB2M r  5'GCAGAGGGGTAGTTCACCTTC-3'

The mutated bases are highlighted in the oligonucleotide sequences (f=forward oligonucleotide sequence, r=reverse nucleotide sequence, M=mutated sequences). The κB binding sites are underlined.

An aliquot (200μl) of each single stranded oligonucleotide, sense and antisense, was mixed with 20μl of NAC (2M, pH 7.0, section 2.2.2) and three volumes (660μl) of ethanol. The solution was precipitated at -80°C for 1 hour and then centrifuged at maximum speed in a microfuge for 10 minutes at room temperature. The pellet was washed twice with 80% ethanol, and then allowed to air dry for 30 minutes. The pellet was resuspended in 40μl water, and the OD of each oligonucleotide was measured at 260nm and 280nm.

2.11: Annealing of oligonucleotides

A volume equal to 400pmol of each oligonucleotide, upper and bottom, (calculated from equation 2.3) was taken, 4μl of 20x annealing buffer was added and made up to 80μl with water. This was heated at 95°C in a water bath for 5 minutes, and cooled slowly to room temperature. The annealed oligonucleotide was stored at -20°C until required.
Equation 2.3:
Absorbance 260nm x dilution factor x 33* = ? mg/ml (A)
(* since for single stranded DNA an OD of 1=33μg)

No of base pairs in oligonucleotide (=24) x MW of 1 base (=330) = MW (in grams)

Therefore for NF-κB oligonucleotide synthesised: 7920g=1mol = 7.9ng=1pmol
For 400pmol = 7.9x400 = 3160ng (=3.2μg) (B)
(B) / (A) = volume of oligonucleotide (μl) required, equal to 400pmL

2.12: DNA Transfections

SW480 cells (1x10⁷ cells) were transfected in serum free DMEM with 1.25pmoles of the plasmid p6NF-κB-tk-LUC (p6NF-κB) (a gift from Dr P Baeuerle, University of Freiberg) and 0.1 pmoles pCMVB, by electroporation. The empty cassette (tk-36-LUK) was used as a negative control. The plasmid pCMVB was cotransfected with the other constructs to normalise luciferase activity to β-galactosidase activity, thus controlling for differences in transfection efficiency.

After transfection, cells were resuspended in DMEM containing 10% FCS and allowed to recover for 5 hours. The medium was then changed to DMEM containing 1% FCS and the cells incubated for a further 24 hours. After serum starving, the cells were exposed to 10ng/ml TNFα for 2 hours, with and without pretreatment for 1 hour with 20μM curcumin. Luciferase and β-galactosidase enzyme activities were measured using Promega assay kits with a Wallach MicroBeta 1450 plate reader or Labsystems iEMS reader, respectively. Luciferase activity was expressed in relative units after normalisation to β-galactosidase.

2.13: Preparation of cytosolic, membrane and nuclear fractions for Western Blot analysis

To study the cellular levels of specific proteins for PKC in response to treatment, cells were resolved into protein fractions; nuclear, membrane and cytosolic. This is important because it both increases the sensitivity and it provides information about the cellular
localisation of PKC since when it becomes activated PKC translocates from the cytosol fraction to the membrane fraction of the cell. Cell fractions of nuclear, membrane and cytosolic components were obtained by homogenisation, gradient centrifugation and sonication (all on ice), as previously described (Grief et al, 1992) with some modifications (Stanwell et al, 1994).

Cells were treated in 90cm² petri dishes (2 dishes/treatment). All solutions used to fractionate the proteins from the HCEC cells and the cell extracts were kept on ice. The cells were washed three times with ice cold wash buffer (section 2.2.4). The buffer was removed and the cells were scraped into 0.2ml swelling buffer (section 2.2.4) and left on ice for 10 minutes. The cells were homogenised with a Citeno homogenisor (Fisons, Loughborough, Leicester). After 6 strokes at speed level three, the homogenate was inspected microscopically to ensure that the cells were disrupted, so the nuclei were free from the cells, but the nuclei were whole. The homogenate was layered over an equal volume of swelling buffer with 25% glycerol (section 2.2) in a 1.5ml tube, to form a dual layer, and centrifuged in a Heraeus minifuge T at 1840rpm for 5 minutes at 4°C, to remove the nuclei and unbroken cells. After centrifugation there were two layers with the nuclei pelleted at the bottom. The nuclear pellet was resuspended in 1ml swelling buffer with 25% glycerol and Triton X-100 (0.1% w/v) (section 2.2.4) and pelleted in a MSE microfuge at 13500 rpm (maximum speed) for 2.5 minutes at 4°C. The resulting nuclear pellet was resuspended in 0.2ml sonication buffer (section 2.2.4) and sonicated using a Bronson sonifier 250 (10 pulses, output level two). The cytosol and membrane fraction (top layer) was separated by ultracentrifugation using a Beckman L7 machine operating at 40000rpm for 35 minutes at 4°C. After centrifugation the supernatant (cytosol fraction) was collected. The pellet (membrane fraction) was washed with 0.5ml swelling buffer (section 2.2.4), and resuspended in swelling buffer with Nonident P-40 (section 2.2.4), and sonicated as for the nuclear fraction.

The protein content of the fractions were measured by the Bradford assay (Bradford, 1976) using Biorad protein assay reagent. The samples were stored at -20°C until further analysis.
2.14: Whole cell protein extract

All solutions and samples were kept on ice. Cell culture dishes were washed 3 times with wash buffer (W3) (section 2.2.5). Excess wash solution was removed and the cells were lysed in homogenisation buffer (H8) (section 2.2.5), by scraping the cells into the solution. The samples were then sonicated for 30 seconds with a Bronson sonifier (duty cycle 10, timer hold, output level 1). The samples were centrifuged at 40000 rpm for 35 minutes at 4°C. The supernatant was collected and the protein content was measured using the method of Bradford (1976), using Biorad solution. The proteins were stored at -80°C.

2.15: Western Blot analysis

Western blot analysis involves separating proteins by electophoresis in a denaturing sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE), transferring them to nitrocellulose electrophoretically and then using a specific primary antibody raised against the protein of interest, and a secondary antibody, for example a biotinylated horseradish peroxidase (HRP) conjugate, to detect binding of the primary antibody.

Complete protein (cell extract) was prepared as described in section 2.14, and used for detection of COX-2 and IκB protein. The amount of protein sample used for each determination is shown in Figure 2.1. PKC protein expression was measured in nuclear, membrane and cytoplasmic protein fractions (section 2.13). Each protein extract was diluted 1:1 in sample buffer (section 2.2.6) and boiled for 5 minutes, prior to use. An aliquot of each protein sample (Table 2.1) was loaded onto an 8%, 10% and 12.5% resolving gel, for PKC, COX-2 and IκB respectively (section 2.2.6), (with dimensions of 10 x 8 x 0.075 cm). This was overlayed with a 5% stacking gel (for PKC and IκB analysis) or 3.5% gel (COX-2) (section 2.2.6). Proteins were subjected to separation at a constant current of 30mA (for two gels) on a Mighty small SE245 gel apparatus (Hoefer Instruments) for approximately 2 hours until the sample buffer dye had reached the bottom of the resolving gel, cooling the gel whilst running. Electrophoretic progress was followed using Rainbow molecular weight markers (14.3-200kD) as recommended by the manufacturers.
The gel and nitrocellulose membrane were immersed in blotting buffer (section 2.2.6) for 15 minutes. The separated proteins were transferred onto nitrocellulose (Hybond-ECL) at 100V at 4°C for 2 hours with a wet transfer method using Bio-Rad Mini Trans-Blot equipment. Following transfer the efficiency of the blot was confirmed by staining the gel overnight with Coomassie blue stain, followed by destaining overnight (section 2.2.6).

<table>
<thead>
<tr>
<th>Amount of protein (µg)</th>
<th>Primary antibody (dilution (v/v))</th>
<th>Secondary antibody (dilution (v/v))</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>40</td>
<td>Goat polyclonal (1:1000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRP-conjugated anti-goat IgG (1:1500)</td>
</tr>
<tr>
<td>IκBα</td>
<td>10</td>
<td>Rabbit polyclonal (1:1000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRP-conjugated anti-rabbit IgG (1:1500)</td>
</tr>
<tr>
<td>PKCα</td>
<td>30</td>
<td>Mouse monoclonal (1:750)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRP-conjugated anti-mouse IgG (1:500)</td>
</tr>
<tr>
<td>PKC zeta</td>
<td>30</td>
<td>Rabbit polyclonal (1:1000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRP-conjugated anti-rabbit IgG (1:1500)</td>
</tr>
</tbody>
</table>

**Table 2.1: Primary and secondary antibody dilutions used for Western blots**

Non-specific binding was blocked by shaking the nitrocellulose membrane in blocking buffer, 10% non-fat dried milk in TBS-T20 (section 2.2.6). For detection of COX-2 protein, the membrane was blocked for 2 hours, with shaking, at room temperature. Prior to detection of PKCα, PKC zeta or IκBα, the membrane was blocked overnight at 4°C. The following procedures were carried out at room temperature on a shaker. After blocking the membrane was washed for 10 minutes and 2 x 5 minutes in TBS-T20. All antibodies were diluted in 1% milk in TBS-T20, with 10ml diluent. For detection of COX-2 protein, the membrane was incubated overnight with a COX-2 specific antibody (Table 2.1). Detection of PKCα, PKC zeta and IκBα was performed by incubation for 2 hours with the indicated dilutions of their specific antibody (Table 2.1). After incubation with the primary antibodies, the blots were washed in TBS-T20 for 2 x 10 minutes, 1 x 7 minutes and 2 x 5 minutes, and incubated with the appropriate species specific HRP linked IgG secondary antibody (Table 2.1), for 1.5 hours. After incubation
the filters were washed as before, with a final wash of TBS alone (no Tween 20) for 5 minutes.

Detection of protein expression was by an ECL method using reagents and hyperfilm from Amersham. The HRP bound specifically to the protein acts as a catalyst for the oxidation of a luminal substrate in the presence of HzO2 and an enhancer to 3-aminophthalate which subsequently emits small but sustained quantities of light. This chemiluminescence is detected using photographic film, giving a measure of the size and amount of protein in the sample. Blots were exposed to film for 2 to 5 minutes and developed using an X-Ograph compact x-2 developer with Kodak GBX developer and fixer. Relative amounts of protein were measured using laser scanning densitometry (Molecular Dynamics).

2.16: Stripping Western Blots

Blots were stripped by incubation with stripping buffer (section 2.2.6) for 30 minutes at 50°C, and washed in TBS-T20 for 2 x 1 minute, and 2 x 10 minutes. The membrane was blocked overnight in 10% milk and reprobed the following day as described.

2.17: Detection of Apoptosis by DNA fragment gel analysis

Internucleosomal cleavage is often associated with apoptosis (Wyllie, 1980) and can be visualised as a DNA ladder by electrophoresis. Solubilisation of the nuclear membrane disrupts the DNA associated with it, allowing it to become a substrate for endogenous endonucleases. When a cell enters the apoptotic pathway there is an endonuclease mediated digestion of the exposed DNA linker regions, between histones in chromatin. DNA is cleaved first into large fragments that vary from 300kb to 50kB in size, then into 180 base pair (bp) multiples. Since the DNA around a histone core is conformationally protected from digestion, and as there is an expanse of 180-200 bp of DNA wrapped around this core, the endonuclease-mediated nucleosome excision is observable as a DNA ladder in agarose gels. The method used below is based on one described by Sorenson et al (1990).
At least $2 \times 10^6$ treated cells were collected, by combining the cells in the culture medium with the monolayer cells (after trypsinisation, section 2.3.1) and pelleted by centrifugation at 1500rpm for 8 minutes at 4°C. The supernatant was carefully removed, to leave the pellets as dry as possible, and stored at 4°C (for a maximum of 2-3 weeks). The cell pellet was resuspended in 15μl sterile water with 6μl of 50mg/ml ribonuclease (RNAse) A (final concentration 10mg/ml) and incubated at room temperature for 25 minutes. Gel loading solution (10.5μl), (section 2.2.7), was added to give a final concentration of 15% Ficoll, 1 x TBE and 0.01% bromophenol blue.

Electrophoresis was performed with a 1.8% agarose gel (electrophoresis grade) in 1 x TBE: The gel was allowed to set before cutting out a strip of the gel just to the negative side of the wells. A digesting gel (0.8% agarose, 2% SDS, 2.5mg/ml proteinase K) was poured into the cut out top. The gel tank was a Hybaid midi gel system fitted with a tube for the recirculation of the electrophoresis buffer.

20μl of each sample was loaded onto the gel, along with standards:
1) 1μg λ DNA digested with *Hind* III
2) 1μg 100bp ladder

both in 11μl TE buffer (section 2.2.3) and 5μl of gel loading solution (section 2.2.7). The gel was ran at 20V for 1 hour and then 90V for 3 hours at 4°C. The gel was rinsed in water, and incubated overnight in TE buffer with RNAse A (20μg/ml), shaking gently at room temperature. After rinsing, the gels were stained for 30 minutes with 0.5μg/ml ethidium bromide, with gentle shaking, and destained in water for 4 hours before viewing with a UV transilluminator and taking a photo of the gel.

2.18: Annexin method

Phosphatidyl serine (PS), a negatively charged phospholipid, is normally restricted to the inner surface of the plasma membrane bilayer. Early in apoptosis, PS is externalised to the outer plasma membrane leaflet, thereby exposing PS to the external environment (Diaz et al, 1999) serving as a trigger for recognition and elimination by macrophages. The process can be monitored using the calcium dependent phospholipid binding protein, annexin V, which preferentially binds to negatively charged phospholipids, such as PS, and is a major component of macrophage and other phagocytic cell
membranes. Annexin V binds to cells with exposed PS early in apoptosis, and continues to be bound through cell death (Andree et al, 1990). PS externalisation is a universal phenomenon, and occurs in most if not all, murine and human cell types undergoing apoptosis independent of the stimulus and proceeds several other apoptotic events (Martin et al, 1995). Annexin V may be conjugated to fluorochromes such as fluorescein isothiocyanate (FITC). This conjugate retains its high affinity for PS, and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Propidium iodide (PI) staining is used to assess plasma membrane integrity in Annexin assays. PI is a fluorescent dye that stains DNA, and does not cross the plasma membrane of cells that are viable or in the early stages of apoptosis, because they maintain plasma membrane integrity. In this method a two-colour flow cytometric approach was used. Using both PI and Annexin V, three cell populations can be identified in scattergrams: PS-/PI- (intact cells), PS+/PI- (cells with PS externalised, but preserved membrane integrity) and PS+/PI+ (cells with compromised membrane).

After treatment, floating cells were collected and the cells remaining attached to the dishes were trypsinised (section 2.3.1), and added to the collected medium. Cells were pelleted using a Sorval TC6 centrifuge (Dupont) at 1000rpm for 5 minutes. The pelleted cells were resuspended in 5mls DMEM. The cells were incubated at 37°C for 10 minutes prior to pelleting by centrifugation at 1000rpm for 5 minutes. The medium was completely removed from the cell pellet with an aspirator. The cell pellet was resuspended in 1ml of annexin buffer, and 2μl annexin protein was added at room temperature. This was quickly mixed by vortexing, and allowed to stand for 10 minutes, before 50μl PI (50μg/ml) was added. The solutions were then transferred to a tube for FACS scan analysis. The samples were run on a flow cytometer with a 488nm Argon laser as the light source.

2.19: Analysis of cell cycle

One of the early events in apoptosis is activation of an endonuclease which nicks DNA preferentially at the internucleosomal (linker) sections. Fixation of cells in ethanol is inadequate to preserve the degraded low molecular weight DNA inside apoptotic cells: this portion of DNA leaks out during subsequent rinsing and staining procedures, and therefore less DNA in these cells stains with any DNA fluorochrome. If the DNA is
stained with PI, which fluoresces under laser light (488 nm), the level of fluorescence emitted from the nucleus, provides an estimate of DNA content. The appearance of cells with DNA staining, lower than that of G1 cells ('sub-G1' peaks) in cell cultures has been considered to be a marker of cell death by apoptosis.

Single cell suspensions were obtained from monolayer cells as follows. The medium in the cell dishes was collected and the monolayer cells trypsinised as previously described (section 2.3.1). Both the adherant and floating cells were pooled and spun down at 1000 rpm for 5 minutes and the cell pellet was resuspended in 200 μl of cold PBS. For cell cycle analysis, the cells were fixed by the vigorous addition of 2 ml of ice cold 70% ethanol/30% PBS to the cells. The cells were left on ice for 30 minutes and then centrifuged at 3600 rpm at 4°C for 10 minutes. The cells were washed twice by resuspending in 1 ml ice-cold PBS, and spun as before. After the second wash, the cell pellet was resuspended in 800 μl of PBS. A 100 μl aliquot of RNase A (1 mg/ml) and 100 μl of PI (312.5 μg/ml) was added to the cell suspension and incubated at 37°C for 30 minutes. The cells were stored at 4°C before measuring their DNA content with a flow cytometer. The cells were analysed by flow cytometry using an argon ion laser tuned to 488 nm (FACScan Becton Dickenson) and the forward and orthogonal light scatter and red fluorescence measured. Cell debris, doublets and fixation artifacts were gated out and the G0/G1, S, G2/M and apoptotic populations recorded. Apoptotic cells were identified as the lower fluorescence peak (sub-G1) on cell number histograms, due to their reduced DNA content.

2.20: Hoechst 33342 staining

The Hoechst dye is a non-intercalating benzimidazole derivative that binds to AT base rich regions of the genome. It emits blue fluorescence when excited by UV light, at about 350 nm, and since it is membrane permeable, it can be used to fluorescently label viable cells. Detection of condensed chromatin allowed detection of apoptotic cells.

The object of the preparative method is to obtain single cells with the minimum degradation of their DNA. Methanol fixation perforates the cell membrane, but preserves most cytoplasmic materials and does not appear to affect dye binding to DNA. Staining with PI facilitated exclusion of non-viable (necrotic) cells from
subsequent analysis. This is a red flurochrome that intercalates between base pairs of ds DNA and RNA without base specificity. PI cannot penetrate viable cells but cells with damaged membranes stain readily, so this dye is often used for differentiating and quantititating viable and dead cells in a given population.

HCEC cells were grown to confluency on sterile coverslips, previously coated with coating medium, in 6 well plates. After exposure to FP-12 or 0.5 or 1mM H$_2$O$_2$ for 4, 8, 16 or 24 hours the medium was removed from the wells and washed with fixative (Methanol: Glacial acetic acid, 3:1). Fresh fixative was added and left for 5 minutes at room temperature, and the cells air dried. Hoechst 33342 (final concentration 1.5µg/ml) and PI (final concentration 5µg/ml) were added to the wells for 15 minutes at room temperature in the dark. The excess stain was washed off by washing the cells three times with PBS and twice with water. Washing with PBS normally removes much of the protein debris. The coverslips were allowed to dry before mounting on slides. After mounting, the number of normal, apoptotic and necrotic cells were quantified by fluorescence microscopy. Ten random fields of view were counted and at least 500 cells were counted in each field. Blue cells with a condensed nuclei were considered apoptotic, and cells stained with a red nucleus were considered as necrotic.

2.21: Statistics

Where appropriate the results are expressed as mean +/- standard error (SE) of n experiments. Statistical analysis was determined by the student's unpaired t test with p< 0.05 considered significant.
CHAPTER 3: INDUCTION AND INHIBITION OF COX-2 mRNA EXPRESSION BY MODEL TUMOUR PROMOTERS AND CHEMOPREVENTIVE AGENTS
3.1: Introduction

There is now considerable evidence from several different experimental systems that COX-2, an inducible isoform of PGHS, may play a role in the genesis of colorectal cancer (Prescott and White, 1996; Oshima et al, 1996). COX-2 gene expression has been shown to be selectively overexpressed in colon carcinogenesis (Kargman et al, 1995). The aim of this work was to investigate the effects of two model colon tumour promoters, FP-12 and TNFα on COX-2 gene expression. TNFα is found elevated in the colonic lumen of patients with inflammatory bowel disease (Braegger et al, 1992), a condition which predisposes them to colon cancer (Gyde et al, 1988). Fecapentaenes have been shown to be tumour promoters in a rat model of colon carcinogenesis (Zarcovik et al, 1993). FP-12 is the most abundant form found in the faeces of individuals who consume a Western diet high in fat and meat (Schiffman et al, 1989b), which is associated with an increased risk for colon cancer.

Numerous epidemiological and clinical studies have demonstrated a 40-50% reduction in the relative risk of colorectal cancer in humans who take NSAIDs on a regular basis (Thun et al, 1993; Marnett, 1992). NSAIDs, such as sulindac, which directly inhibit COX-2, caused regression of adenomatous polyps in FAP patients (Giardiello et al, 1993). The mechanism for the chemopreventive effects of NSAIDs is assumed to be due to direct COX-2 enzyme inhibition. Chronic use of these drugs has been linked to unwanted side effects, such as gastrointestinal mucosal lesions, due to a concomitant inhibition of COX-1 (Eberhart and DuBois, 1995). Therefore one aim was to investigate other agents which could selectively inhibit COX-2 gene expression, particularly those that form part of the diet, as more desirable chemopreventive agents. Curcumin is one such agent, and has been shown to possess potent chemopreventive activity against colon cancer in animal models of this disease (Rao et al, 1993a), but its mechanism of action is not well documented. Therefore the effects of this agent on COX-2 mRNA induction by tumour promoters was investigated. The effect on COX-2 mRNA induction of two other dietary polyphenols, CAPE, a structural analogue of curcumin, and resveratrol, were also investigated, both of which could also be important in colon cancer chemoprevention (Rao et al, 1995c). To determine if the effect of these agents, curcumin, CAPE and resveratrol on COX-2 gene expression, was due to their antioxidant properties, a classical antioxidant, NAC was also investigated. NAC has
been shown to have chemopreventive activity in rat and mouse models of cancer (DeFlora et al, 1991), is readily taken up by cells, and is rapidly converted to glutathione, acting as an intracellular free radical scavenger. Finally the effect of these agents on COX-2 protein levels was investigated.

3.2: RNA quantification and validation of RT-PCR

Total RNA was isolated from HCEC cells using TRIZOL® reagent as described in section 2.5. The absorbance at 260 and 280 nm was measured for each sample and table 3.1 shows typical yields of total RNA. Total RNA was calculated using equation 2.2 (section 2.5).

**Table 3.1: Total RNA yield from HCEC cells after Trizol extraction**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ABSORBANCE</th>
<th>RATIO (260/280)</th>
<th>TOTAL RNA (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>260nm</td>
<td>280nm</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.0967</td>
<td>0.0629</td>
<td>1.5</td>
</tr>
<tr>
<td>FP-12 (20µM)</td>
<td>0.0983</td>
<td>0.0653</td>
<td>1.5</td>
</tr>
<tr>
<td>FP-12 (20µM) + curcumin (20µM)</td>
<td>0.0800</td>
<td>0.0501</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Absorbances shown are for a 1 in 200 dilution of the isolated RNA and are representative of all isolations.

To study the mRNA levels of the COX-2 gene, initially Northern blot analysis was performed. This however proved unsatisfactory due to a lack of sensitivity, and the need for a large amount of RNA (20-30µg). Instead a semi-quantitative RT-PCR analysis of COX-2 mRNA levels was performed, according to the method of Hla and Maciag (1991) requiring only 1µg of RNA as starting material. The RNA was dissolved in 50µl DEPC water and the total amount of RNA obtained from Trizol extraction from 2 x 10^7 cells, was between 30-40µg.
Figure 3.1: Validation of RT-PCR method to measure COX-2 gene expression. RNA isolated from HCEC cells exposed to TNFα for 2 hours, or control, unexposed cells were amplified in an RT-PCR reaction with primers for COX-2 and GAPDH, for increasing numbers of cycles, from 18-30 PCR cycles. (A) representative agarose gel of COX-2 and GAPDH RT-PCR products from control HCEC cells or cells exposed to 10ng/ml TNFα generated after 18-30 cycles of amplification. Band strength was quantified with a laser densitometer, and shown as a graph of cycle number against mRNA level (arbitrary units) for (B) COX-2 and (C) GAPDH products.
Total RNA was amplified using the appropriate primers for COX-2 and GAPDH in an RT-PCR reaction as described in section 2.6. A preliminary experiment was performed to determine the number of cycles to use in the PCR reaction, since during PCR the amount of product initially increases exponentially, but then the rate slows and finally plateaus. It is important to use a cycle number in PCR reactions before saturation occurs. An RNA sample with expected low levels of COX-2 (i.e., RNA extracted from control, untreated cells) and RNA from TNFα (10ng/ml) exposed cells (high COX-2 mRNA) was amplified for increasing numbers of cycles, between 18-30 cycles. The PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and visualised using UV light as described in section 2.7. From Figure 3.1 a cycle length for the PCR method was chosen of 25 cycles, since this confirmed that the amount of amplified product by this method, was in the linear range of amplification with respect to input RNA for both COX-2 and GAPDH primers. The band strength determined under these conditions would then be directly proportional to the amount of input RNA. Expression was quantified for all RT-PCR results using a laser densitometer (Molecular Dynamics).

3.3: Effect of exposure of HCEC cells to TNFα or FP-12 on COX-2 mRNA expression

HCEC cells were exposed to TNFα (1ng/ml) or FP-12 (20μM) for various times between 15 minutes to 24 hours to determine at what time point there was maximal induction of COX-2 mRNA expression. RNA was extracted from the cells as previously described (section 2.5), prior to RT-PCR analysis of COX-2 mRNA expression (section 2.6). The results of all RT-PCR reactions have been corrected for any loading differences between starting RNA preparation by measuring GAPDH expression for each sample to normalise COX-2 band densities, in parallel PCR reactions. GAPDH is a constitutively expressed gene and it is amplified in the PCR reaction in the same way as COX-2 to act as an internal standard for loading differences between samples. In this semi-quantitative method the COX-2 mRNA expression was normalised to 1 in the control HCEC cells, and the RNA level in treated samples was expressed relative to control cells. The results, in Figure 3.2 show that COX-2 mRNA induction started to increase in response to FP-12 and TNFα within 1 hour of incubation with these agents, and a maximum fold increase in message levels was seen after 1 to 2 hour stimulation.
COX-2 mRNA (fold increase relative to control)

Time of exposure to TNFα (hours)

- Control
- 15 mins
- 30 mins
- 1 hour
- 2 hours
- 3 hours
- 6 hours
- 24 hours
Figure 3.2: Kinetics of COX-2 mRNA expression following exposure to TNFα or FP-12. HCEC cells were exposed to TNFα (1ng/ml) or FP-12 (20μM) for various times, between 15 minutes to 24 hours. RNA was extracted from the cells and COX-2 mRNA expression analysed by RT-PCR. An aliquot of the PCR products were run on a 1% agarose gel and visualised using UV light (A) gel for cells exposed to TNFα (1ng/ml) and (C) gel for cells exposed to FP-12 (20μM). Time of exposure was plotted against COX-2 mRNA (expressed as fold increase relative to control cells), and normalised with GAPDH, (B) TNFα exposed cells and (D) FP-12 exposed cells. The results shown are representative of two experiments performed on two different occasions.
Figure 3.3: COX-2 mRNA levels after exposure of HCEC cells to various concentrations of TNFα or FP-12. HCEC cells were exposed to various concentrations of TNFα or FP-12 for 2 hours prior to RNA extraction and analysis of COX-2 mRNA expression by RT-PCR. (A) The PCR products for GAPDH and COX-2 were run on an agarose gel. The band densities were quantified using a densitometer (Molecular Dynamics) and results expressed as fold increase in COX-2 mRNA relative to control cells, and normalised for loading with GAPDH; (B) TNFα exposed cells and (C) FP-12 exposed cells. The results in (B) and (C) are the mean +/- standard deviation of 3 separate experiments performed on different cell cultures on different occasions.
with FP-12 and 2 to 3 hours with TNFα. COX-2 induction was not further increased up to 24 hours of stimulation, and its level returned to control levels after exposure to either agent for 24 hours. The result suggests that the effects of these agents on COX-2 mRNA expression were mediated at the level of transcription.

COX-2 expression was determined after exposure of HCEC cells to increasing doses of FP-12 or TNFα for 2 hours. The concentrations of the two agents that were used were determined from estimations of levels that could be achieved in vivo. Both TNFα (0.1-10ng/ml) and FP-12 (1-40μM) caused a dose dependent induction of COX-2 mRNA levels, (Figure 3.3B and Figure 3.3C respectively). TNFα (0.1-10ng/ml) caused a 2 to 6 fold induction of COX-2 mRNA, (Figure 3.3B). A similar effect was seen with FP-12 (1-40μM), but COX-2 mRNA was increased by up to 8 fold relative to unstimulated cells (Figure 3.3C). Based on these results, concentrations of 1 or 10ng/ml TNFα and 20μM FP-12 were used for investigating the effects of NSAIDs, curcumin, CAPE and resveratrol on COX-2 gene expression.

3.4: Effect of chemopreventive agents on TNFα and FP-12 induced COX-2 gene expression

3.4.1: Sodium Salicylate

HCEC cells were pretreated for 1 hour with 10 or 20mM sodium salicylate prior to 10ng/ml TNFα or 20μM FP-12 for 2 hours. RNA was extracted from the cells and RT-PCR was performed (section 2.5 and section 2.6 respectively). TNFα and sodium salicylate alone both caused an approximate 6 fold increase in COX-2 mRNA relative to control cells (Figure 3.4A). Contrary to expectation, sodium salicylate (10 or 20mM) increased COX-2 expression with TNFα, (Figure 3.4A) from 6.2 fold for TNFα alone to 13.3 and 14.9 fold above expression seen in unstimulated cells after pretreatment with 10 and 20mM sodium salicylate respectively. It appears that there was an additive effect in HCEC cells exposed to both sodium salicylate and TNFα, relative to that seen after exposing the cells to either of these agents alone. Similar results were seen in cells exposed to FP-12 for 2 hours after pretreatment with 10mM sodium salicylate for 1 hour (Figure 3.4B). An additive effect on COX-2 mRNA expression was observed in
Figure 3.4: COX-2 mRNA levels after exposure of HCEC cells to TNFα or FP-12 in the absence or presence of sodium salicylate. HCEC cells were exposed to 10 or 20 mM sodium salicylate for 1 hour prior to (A) TNFα (10 ng/ml) or (B) FP-12 (20 μM) for 2 hours. RNA was extracted from the cells and RT-PCR performed. The results are expressed as fold increase in COX-2 mRNA relative to control cells, and normalised with GAPDH. The results shown are the mean +/- range of 2 experiments performed on different cell cultures on different occasions.
cells exposed to both FP-12 and sodium salicylate, compared to exposure of either agent alone.

3.4.2: Curcumin

HCEC cells were exposed to 1 or 10ng/ml TNFα for 2 hours after pretreatment with curcumin (10-40μM) for 1 hour. RNA was extracted from the cells and RT-PCR was performed with primers for COX-2 and GAPDH (sections 2.5 and 2.6 respectively). Curcumin caused a dose dependent reduction in COX-2 mRNA in cells exposed to both 10 and 1ng/ml TNFα, (Figure 3.5 A and B respectively). Curcumin (10μM) had no significant effect on COX-2 mRNA induction by 10ng/ml TNFα, there was a slight reduction with pre-treatment with 20μM curcumin and an approximate 35% reduction in COX-2 mRNA expression in cells pretreated with 40μM curcumin prior to 10ng/ml TNFα (Figure 3.5A). This was significantly different from cells exposed to TNFα alone, as determined by analysis of variance (student’s t test) (* = p<0.05). Incubation of HCEC cells with increasing concentrations of curcumin (10-40μM) for 1 hour prior to stimulation with 1 ng/ml TNFα for 2 hours also resulted in a dose dependent reduction in the levels of the COX-2 transcript (Figure 3.5B). A significant (p<0.05) 50% reduction in COX-2 expression was observed after pretreatment with a dose as low as 10μM curcumin, and at higher doses of 20 and 40μM curcumin the COX-2 mRNA was reduced by about 90%, similar to mRNA expression in unstimulated cells. This was highly significant (p<0.01), when cells pretreated with curcumin prior to 1ng/ml TNFα differed significantly from cells exposed to TNFα alone. Curcumin (20μM) also caused a significant, approximate 60% reduction of COX-2 mRNA expression induced by FP-12 (20μM); (Figure 3.5C). Curcumin alone, had no effect on COX-2 mRNA expression compared to unstimulated cells.

3.4.3: CAPE

To determine to what extent the inhibitory effects of curcumin on COX-2 mRNA levels was due to its antioxidant activity, the effects of a structurally related antioxidant and chemopreventive agent, CAPE, was investigated. HCEC cells were pretreated for 1 hour with CAPE, prior to treatment with TNFα, 1 or 10ng/ml, or FP-12 (20μM), for 2 hours.
B

COX-2 mRNA (fold increase relative to control)

![Graph showing COX-2 mRNA levels with different treatments.](image_url)
Figure 3.5: COX-2 mRNA levels after exposure of HCEC cells to TNFα or FP-12 in the absence or presence of curcumin. HCEC cells were pretreated for 1 hour with various concentrations of curcumin, prior to TNFα (1 and 10ng/ml) (A and B respectively) or FP-12 (20µM) (C), for 2 hours. RNA was extracted from the cells and COX-2 mRNA expression analysed by RT-PCR. The band densities were quantified using a densitometer and results expressed as fold increase in COX-2 mRNA relative to control cells, and normalised for loading with GAPDH. Statistical significance was determined (* p< 0.05 and ** p< 0.01) by analysis of variance (student’s t test), where cells pretreated with curcumin prior to TNFα or FP-12 treatment differ significantly from treatments with TNFα or FP-12 alone. Results are the means +/- standard deviation of 3 measurements performed on different cell cultures on 3 different occasions.
COX-2 mRNA (fold increase relative to control)

- Control
- CAPE (20μM)
- TNFα (1ng/ml)
- CAPE (10μM) + TNFα
- CAPE (20μM) + TNFα
- CAPE (40μM) + TNFα
- CAPE (20μM)
- TNFα (10ng/ml)
- CAPE (10μM) + TNFα
- CAPE (20μM) + TNFα
- CAPE (40μM) + TNFα
Figure 3.6: Effect of CAPE pretreatment on COX-2 mRNA levels after exposure of HCEC cells to TNFα or FP-12. HCEC cells were pretreated for 1 hour with various concentrations of CAPE, prior to TNFα (1 or 10ng/ml) (A and B) or (C) FP-12 (20μM), for 2 hours. RNA was extracted from the cells and COX-2 mRNA expression analysed by RT-PCR. The results are expressed as fold increase in COX-2 mRNA relative to control cells, and normalised for loading with GAPDH. The results presented are the mean +/- range of three separate experiments.
COX-2 expression was determined after RNA extraction from the cells followed by RT-PCR. CAPE (10 and 20μM) had no inhibitory effect on COX-2 mRNA levels in cells treated with 10ng/ml TNFα, and in cells pretreated with 40μM CAPE there was an increase in COX-2 mRNA above that seen in cells treated with TNFα alone, (Figure 3.6A). When HCEC cells were exposed to TNFα 1ng/ml, after pretreatment with CAPE there was only a small reduction in COX-2 mRNA expression compared to exposure to TNFα alone, and a similar effect was seen with 10, 20 and 40μM CAPE, suggesting that the effect was not dose dependent, (Figure 3.6B). Pretreatment of HCEC cells with 40μM CAPE prior to FP-12 treatment also had no inhibitory effect on the induction of COX-2 mRNA expression by FP-12 (Figure 3.6C). CAPE (20μM) alone had no effect on COX-2 mRNA, compared to the expression seen in the control cells.

3.4.4: Resveratrol

Resveratrol, is another polyphenolic compound. Again cells were exposed to resveratrol (10-40μM), for 1 hour prior to exposure to TNFα (1ng/ml) or FP-12 (20μM) for 2 hours. RNA was extracted from the cells and COX-2 mRNA expression measured by RT-PCR (section 2.5 and 2.6 respectively). TNFα and FP-12 both caused a marked 5-6 fold induction of COX-2 mRNA expression above that seen in unstimulated cells after 2 hours exposure, (Figure 3.7A and B respectively). Pretreatment with resveratrol (10, 20 or 40μM) caused an approximate 40% inhibition of COX-2 mRNA in cells subsequently treated with both TNFα or FP-12, but dose dependency was not seen. This is similar to the results seen with pretreating HCEC cells with CAPE, prior to TNFα (1 ng/ml), (Figure 3.6B), where no dose dependent inhibitory effect was seen. Resveratrol alone did not increase COX-2 mRNA expression above that seen in control cells.

3.4.5: NAC and mannitol

To further assess the possible antioxidant effects of curcumin on COX-2 gene expression the effect of the classical antioxidants NAC and mannitol on COX-2 mRNA induction by the tumour promoters was investigated. HCEC cells were pretreated with 5mM NAC overnight, prior to TNFα (1ng/ml) or FP-12 (20μM), for 2 hours. Figure 3.8 shows that NAC had no inhibitory effect on COX-2 mRNA levels by TNFα (A) or FP-
Figure 3.7: COX-2 mRNA levels after exposure of HCEC cells to TNFα or FP-12 in the absence or presence of resveratrol. HCEC cells were pretreated for 1 hour with various concentrations of resveratrol, prior to TNFα (1ng/ml) (A) or FP-12 (20μM) (B) for 2 hours. RNA was extracted from the cells and COX-2 mRNA expression analysed by RT-PCR. The results are expressed as fold increase in COX-2 mRNA relative to control cells, and normalised for loading with GAPDH. The results are the mean +/- range of two separate experiments performed on different cell cultures.
Figure 3.8: COX-2 mRNA levels after exposure of HCEC cells to TNFα in the absence or presence of N-acetyl cysteine (NAC) or mannitol. HCEC cells were pretreated with 5mM NAC, overnight, or 2mM mannitol, for 45 minutes prior to TNFα (A) or FP-12 (B) for 2 hours. RNA was extracted from the cells and RT-PCR performed. The results are expressed as fold increase in COX-2 mRNA relative to control cells, and normalised with GAPDH. The results shown are from one experiment, representative of two experiments, performed on different cell cultures on two different occasions.
12 (B); it increased COX-2 mRNA above that seen with FP-12 alone. NAC alone also caused an increase in COX-2 mRNA by approximately 2 fold, compared to control cells (no treatment). HCEC cells pretreated for 45 minutes with 2mM mannitol, a scavenger of hydroxyl radicals, prior to FP-12, like NAC, showed no inhibitory effect on the induction of COX-2 mRNA.

3.5: Effect of TNFα and FP-12 on COX-2 Protein levels.

To determine if the suppression of COX-2 mRNA by curcumin was reflected in the translation of protein encoded by this mRNA, total protein was extracted from the HCEC cells (section 2.14) and COX-2 protein levels were analysed by Western blot with antibodies specific to COX-2 (section 2.15). Western blot analysis revealed that exposure of HCEC cells to TNFα, 1ng/ml and 10ng/ml, and FP-12 20μM and 40μM, for 5 hours caused an increase in COX-2 protein, above that seen in control (unstimulated cells), (Figure 3.9). This induction of COX-2 protein by TNFα and FP-12, was reduced by pretreatment with curcumin (40μM) for 1 hour, (Figure 3.9). In addition, H₂O₂ was shown to be a strong inducer of COX-2 protein, stronger than that seen with TNFα or FP-12, and this induction was inhibited by one hour pretreatment of HCEC cells with 40μM curcumin.

3.6: Discussion

Considerable evidence exists from several different model systems that COX-2 may play a role in the pathogenesis of colon cancer. In these studies COX-2 is upregulated in transformed cells and in tumours (Kargman et al, 1995). COX-2 overexpression may be due to exposure of colon epithelial cells to endogenous factors influenced by the diet. Investigations into the role of COX-2 in cancer have suggested that chronic activation of this enzyme may be important in the colon, where there is convincing evidence to suggest that the inhibition of COX-2, can limit the progression of colorectal cancer (Oshima et al, 1996). Therefore chemopreventive strategies have focused on inhibitors of COX-2 enzyme activity. The COX-2 enzyme has both cyclooxygenase and peroxidase activities (Marnett, 1992; Smith et al, 1991). Apart from its importance in prostaglandin synthesis, the peroxidase function contributes to the activation of procarcinogens (Eling et al, 1990). NSAIDs inhibit the cyclooxygenase, but not the
Figure 3.9: Effect of curcumin on COX-2 protein expression induced by TNFα, FP-12 or H2O2 in HCEC cells. HCEC cells were exposed to 40μM curcumin for 1 hour prior to TNFα (1 or 10ng/ml), FP-12 (20μM) or H2O2 (1mM), for 5 hours. Protein was extracted from the cells, and COX-2 protein levels analysed by Western blots. Lanes 1 and 7: control, lane 2: TNFα (1ng/ml), lane 3: TNFα (10ng/ml), lane 4: curcumin (40μM) + TNFα (1ng/ml), lane 5: curcumin (40μM) + TNFα (10ng/ml), lane 6 and 10: curcumin (40μM), lane 8: FP-12 (20μM), lane 9: curcumin (40μM) + FP-12 (20μM), lane 11: H2O2 (1mM), lane 12 curcumin (40μM) + H2O2 (1mM). Quantification of COX-2 protein levels by densitometry are shown in brackets, where values are shown as fold increase relative to control cells. The results shown are representative of two separate experiments, performed on different cell cultures on two different occasions.
peroxidase activity of COX (Mizuno et al, 1982) which potentially limits the effectiveness of this therapy. Because COX-2 transcription is increased in transformed cells (Subbaramaiah et al, 1996; Kutchera et al, 1996), these cells may have functional enzyme activity despite NSAID treatment. Therefore an equally important strategy may be to identify compounds that suppress the expression of COX-2 at the message level (Mestre et al, 1997). The effects of three candidate chemopreventive agents on COX-2 gene induction by the two endogenous tumour promoters, FP-12 and TNFα, was investigated in the present study.

A semi-quantitative RT-PCR method was adapted to measure the COX-2 transcript, since it may be difficult to detect COX-2 mRNA by Northern blot analysis, due to its absence or low abundance in normal colon tissue. Unstimulated HCEC cells expressed small but detectable levels of COX-2 mRNA using this methodology. Any detectable, but low levels of COX-2 mRNA (or protein) in HCEC cells could be due to a small percentage (2%) of serum in the DMEM medium, since serum itself can induce COX-2 expression (O'Banion et al, 1991). The expression of COX-2 mRNA and/or protein has been shown to be induced in a variety of cells following addition of serum, for example in src-transformed chicken fibroblasts (Xie et al, 1991; O'Neil and Ford-Hutchinson, 1993). Both TNFα and FP-12 induced a rapid and transient increase in COX-2 mRNA levels in HCEC cells. It could be suggested from the results of the present study that TNFα and FP-12 caused a direct transcriptional effect on the COX-2 gene, since an increase in COX-2 mRNA levels was seen within 30 minutes following treatment. The time profile of the response to these agents was different with a maximum COX-2 mRNA response detected between 2 and 3 hours after stimulation with TNFα and 1 to 2 hours after stimulation with FP-12. This difference may reflect distinct intracellular signalling pathways for the effect on COX-2 mRNA expression of these two agents. After both treatments COX-2 mRNA levels declined rapidly and returned to control levels within 24 hours in the constant presence of the stimuli. This agrees with other studies on COX-2 mRNA induction at the transcriptional level, where it was reported that COX-2 mRNA induction was rapid and transitory (Perkins and Kniss, 1997). This is a typical time profile of induction of an early response gene, defined as a gene whose expression is low (or absent) in quiescent cells but can be rapidly activated by a variety of extracellular stimuli, including growth factors, cytokines and tumour promoters (Herschman, 1991). Regulation of expression is rapid occurring from minutes to hours.
following exposure to the stimuli, with an equally rapid return of expression to baseline levels. The induction of COX-2 mRNA expression by TNFα and FP-12 was concentration dependent; TNFα (0.1-10ng/ml) caused a dose dependent, 2 to 6 fold induction in COX-2 mRNA expression and FP-12 (1-40µM) caused a 2 to 8 fold increase in COX-2 mRNA expression above that detected in control cells. Therefore both TNFα and FP-12 induce a direct time and dose dependent increase in the expression of COX-2 mRNA. The doses that were used to investigate the effect of TNFα on COX-2 gene expression, were doses of TNFα which could be observed in vivo. In inflammatory disease, circulating TNFα levels can increase markedly, with serum levels as high as 2.8ng/ml reported for patients with rheumatoid arthritis (Ureugdenhil et al, 1992) and 6ng/ml for some cancer patients (Nakashima et al, 1995).

FP-12 has been reported to demonstrate tumour promoting activity in a rat colon carcinogenesis model, using N-methyl-N-nitrosurea as the initiating agent (Zarkovic et al, 1993), although no mechanism for this has been reported. The results of the present study demonstrate that FP-12 can induce the expression of COX-2 in HCEC cells, a normal colon epithelial cell line, suggesting that FP-12 could act as a tumour promoter in this cell culture model. Zhang et al (1998) investigated the induction of COX-2 expression by bile acids, and indicated as with FP-12, that COX-2 induction could be the mechanism of tumour promotion of these diet-derived agents. Zhang et al (1999) also reported that bile acids increase COX-2 transcription and activity. The fecapentaene concentration in human stool has been calculated to be between 0.3 and 30µM (Schiffman et al, 1989a), and therefore exposing HCEC cells to doses of FP-12 below 40µM is likely to be similar to concentrations that are observed in vivo.

Exposure of HCEC cells to sodium salicylate (10 or 20mM) for 1 hour prior to TNFα (10ng/ml) or FP-12 (20µM) for 2 hours, did not cause a reduction in COX-2 gene expression; it increased the induction of COX-2 gene expression compared to exposure to TNFα or FP-12 alone. Sodium salicylate on its own induced COX-2 expression, to about the same extent as TNFα or FP-12 (a 6 to 7 fold increase in COX-2 mRNA expression compared to control HCEC cells). It appears that a combination of either TNFα or FP-12 and sodium salicylate caused an additive effect on COX-2 mRNA expression. In these studies the effect of sodium salicylate was investigated rather than
aspirin because of the difficulty in maintaining a continuous exposure to aspirin, which is extremely labile, and hydrolyses completely to salicylic acid within minutes after introduction into the tissue culture medium (Rumble and Roberts, 1981). Also aspirin is rapidly deacetylated in blood to form salicylic acid (Xu et al, 1991). The finding that treatment with sodium salicylate, an inducer of apoptosis, caused induction of COX-2 mRNA was unexpected, since aspirin and sodium salicylate have been reported to decrease COX-2 enzyme activity. The increased COX-2 message, above that seen in control HCEC cells may be non-functional, since Lu et al (1995) have found that the predominant form of COX-2 induced by these drugs was partially spliced and non-functional RNA. These authors also suggested that salicylic acid showed negligible inhibition of COX-2 in mammalian or chicken fibroblasts. O’Sullivan et al (1993) reported suppression of COX-2 expression in rat alveolar macrophages induced with LPS by dexamethasone, but not by aspirin. Similar results were observed in murine fibroblasts activated with phorbol esters (Kujubu and Hershman, 1992). Barrios-Rodiles et al (1996) showed that NSAIDs did not inhibit COX-2 expression but only inhibited COX-2 enzymatic activity, in U937 cells. Mitchell et al (1997) have also reported that salicylic acid inhibited COX-2 activity, but did not decrease COX-2 mRNA or protein induced by IL1-β in A549 cells. The mechanism whereby NSAIDs influence the expression of COX-2 is unknown. However, one or more COX produced products, such as prostaglandins, may repress COX expression in a negative feedback loop. This is a possible explanation for the induction of COX-2 expression by sodium salicylate, where the removal of this negative feedback by NSAID treatment could result in COX-2 induction (Lu et al, 1995). There are also some reports that COX-2 expression can be increased by prostaglandins, such as PGE2 in a positive feedback mechanism (Raisz et al, 1993). Another explanation of why sodium salicylate did not reduce COX-2 gene expression could be that these drugs are not specific COX-2 inhibitors, but may be more selective COX-1 inhibitors (Mitchell et al, 1993). However more recently, Xu et al (1999) have reported that salicylate inhibited COX-2 expression. The concentration of sodium salicylate used in the present study was chosen from the concentration (1-5mM) that has been measured in the serum of patients treated with aspirin and sodium salicylate for chronic inflammatory diseases. However, even if sodium salicylate can inhibit prostaglandin synthesis, it may not necessarily be able to inhibit the expression of COX-2 itself.
Exposing HCEC cells to curcumin (10-40μM) for 1 hour prior to TNFα (1 or 10ng/ml) caused a significant, dose dependent decrease in COX-2 mRNA expression. Pretreatment with 20 and 40μM curcumin prior to 1ng/ml TNFα reduced the COX-2 expression back to that seen in control, unexposed HCEC cells, and the reduction was highly significant (p<0.01). Pretreatment for 1 hour with 20μM curcumin prior to FP-12 (20μM) also caused a significant (p<0.05), 50% reduction in COX-2 mRNA expression compared to control cells from 5-6 to 2-3 fold above that observed in control HCEC cells. This inhibitory effect of curcumin on COX-2 mRNA expression could be because curcumin specifically targets COX-2. However, as the effects of curcumin on COX-1 expression was not measured it is not possible to say from this data whether curcumin specifically inhibits expression of the COX-2 isoform of PGHS. Zhang et al (1999) have recently reported that curcumin (5-20μM) caused inhibition of bile acid and phorbol ester mediated induction of prostaglandin synthesis, by inhibiting COX-2 expression and subsequent activity.

It has been previously reported that resveratrol inhibits the enzymatic activity of COX-1 and to a lesser extent the hydroperoxide activity of COX-2 (Jang et al, 1997). Pretreatment of HCEC cells with resveratrol only produced an approximate 40% inhibition of COX-2 mRNA expression induced by 1ng/ml TNFα and 20μM FP-12, and all concentrations of resveratrol (10-40μM) reduced the COX-2 mRNA expression to the same extent, indicating no dose dependency. Jang and Pezzuto (1998) reported that pretreatment of female CD-1 mice for 30 minutes with resveratrol (1-25μM), similar to concentrations that were used in the present study, prior to TPA (10nmol) for 4 hours also did not alter the expression of COX-2 induced by TPA. Conversely, Subbaramaiah et al (1998) reported that resveratrol (2.5-20μM) inhibited COX-2 mRNA induced by 50ng/ml PMA in a dose dependent manner when measured by Northern blot analyses. In addition Subbaramaiah et al (1998) observed with transfection studies that resveratrol suppressed TPA mediated induction of COX-2 promoter activity. Since they reported that resveratrol suppresses the activation of COX-2 gene expression by inhibiting a signal transduction pathway involving PKC, it is possible that resveratrol could not inhibit COX-2 induction by FP-12 because PKC may not be the only important pathway of cellular signalling by FP-12. Differences in findings between groups, suggests that the effect of resveratrol on COX-2 gene expression may be species or test system specific.
Pretreatment of HCEC cells with 10, 20 or 40μM CAPE, prior to TNFα or FP-12 showed no inhibitory effect on COX-2 mRNA expression, compared to exposure to TNFα (1 or 10ng/ml) or FP-12 (20μM) alone, and similar expression of COX-2 was observed with all concentrations of CAPE. CAPE alone had no effect on constitutively expressed COX-2. Pretreatment of HCEC cells with the classical antioxidant NAC (5mM overnight) or mannitol (2mM, 45 minutes), a scavenger of hydroxyl radicals, prior to TNFα (1ng/ml) or FP-12 (20μM) did not cause down-regulation of COX-2 mRNA expression. Pretreatment of HCEC cells with NAC prior to FP-12 resulted in an increase in COX-2 mRNA expression from 3.5 fold with FP-12 alone to 4.5 fold, with both NAC and FP-12, above that detected in control cells. Exposure of HCEC cells to NAC-alone, caused a 2 fold increase in COX-2 expression compared to control cells, whereas mannitol alone had no effect. This suggests that ROS were not the major contributors in the signalling pathway to activate COX-2 mRNA induction by TNFα and FP-12, since NAC attenuates oxygen radical formation at concentrations of 10mM and below (De Flora et al, 1996). In addition the inhibition of COX-2 mRNA expression in cells by curcumin pretreatment, prior to TNFα or FP-12, may be independent of its antioxidant properties.

Changes observed at the message level on COX-2 expression by TNF-α and FP-12 in HCEC cells was also observed at the protein level, since the results of the RT-PCR are consistent with the Western blot data. This suggests that an increase in the expression of COX-2 mRNA also leads to an increase in COX-2 protein, in the model system utilised in the present study. In addition the ability of curcumin to suppress the induction of COX-2 mRNA by TNFα, was reflected in its inhibition of the translation of protein encoded by this mRNA.

In conclusion, the induction of COX-2 mRNA expression by FP-12 and TNFα could be important in colon carcinogenesis since COX-2 overexpression inhibits apoptosis, and increases the invasiveness of malignant cells (Tsujii and DuBois, 1995). Since curcumin has been shown to inhibit COX-2 mRNA expression it may be a good candidate for a chemopreventive agent against colon carcinogenesis in humans. It may be important to determine whether combining agents that suppress the transcription of the COX-2 gene, such as curcumin with agents that inhibit COX activity, for example NSAIDs is more effective than either alone in preventing colon cancer.
CHAPTER 4: ROLE OF NF-κB ACTIVATION IN THE MECHANISM OF TUMOUR PROMOTION BY FP-12 AND TNFα
4.1: Introduction

Overexpression of COX-2 in tumour cells is thought to be due to alterations in transcriptional control (Kutchera et al, 1996), but the transcription factors involved have yet to be determined. The promoter region of the COX-2 gene contains consensus binding sites for several transcription factors, including two NF-κB sites (Appleby et al, 1994). It is conceivable that NF-κB could be implicated in COX-2 mRNA induction by FP-12 and TNFα. TNFα is a potent activator of NF-κB in many cell types (Schutze et al, 1995) and FP-12 has been shown to induce oxidative stress in cells, and act as a cofactor for PKC (Hoshina et al, 1991) which in turn can activate NF-κB.

NF-κB is a transcription factor that regulates the expression of many genes especially those involved in the immune and inflammatory responses (Baeuerle and Baltimore, 1991). NF-κB is known to play a role in controlling the transcriptional activity of the COX-2 gene. COX-2 overexpression caused by hypoxia in HUVEC and by IL-1β in rheumatoid synoviocytes has been shown to be mediated by NF-κB (Schmedtje et al, 1997; Crofford et al, 1997). The aim of the present study was to investigate whether NF-κB could be involved in COX-2 induction in HCEC cells by TNFα and FP-12, and its inhibition by chemopreventive agents.

In resting cells NF-κB exists predominantly as an inactive complex, bound to an inhibitor protein IκB in the cytoplasm (Beg and Baldwin, 1993). It is believed that all stimuli converge into a common pathway to activate NF-κB involving phosphorylation and subsequent degradation of IκB. After degradation of IκB, NF-κB is released and translocates to the nucleus and binds to gene promoter regions to activate gene transcription (Verma et al, 1995). Agents that interfere with the signalling mechanisms governing transcription of COX-2 should inhibit tumourigenesis. Chemopreventive agents may act by blocking the induction of COX-2 gene expression by inhibition of signalling via NF-κB. A number of antioxidative agents have been reported to suppress the activation of NF-κB in vitro and in vivo (Schreck et al, 1991). Curcumin has been shown to block many reactions in which NF-κB plays a role (Singh and Aggarwal, 1995) and has been shown to be a potent inhibitor of NF-κB activation, possibly by preventing IκB degradation and nuclear translocation of NF-κB. The precise signalling
pathways that mediate activation of NF-κB have yet to be completely determined, but a serine/threonine kinase that leads to the phosphorylation of IκB, called the IκB kinase (IKK), consisting of 2 subunits, IKKα and IKKβ have recently been cloned (DiDonato et al, 1997). Phosphorylation by PKC could be important for the removal of NF-κB from IκB and through the augmentation of the transactivation of NF-κB itself (Schmitz et al, 1995), by some stimuli. Curcumin has been reported to inhibit phorbol ester-induced PKC activity in mouse fibroblast cells (NIH3T3) (Liu et al, 1993). Curcumin has also been shown to be a kinase inhibitor by inhibiting EGF receptor autophosphorylation (Korutla et al, 1995). It is possible that curcumin may act on signalling kinases that regulate IκB stability.

4.2: Effect of exposure of HCEC cells to TNFα or FP-12 in the presence or absence of curcumin, CAPE or resveratrol, on NF-κB DNA binding to a consensus NF-κB oligonucleotide in EMSAs

To assess the effect of a number of agents on the activity of the transcription factor NF-κB, HCEC cells were pretreated for 1 hour with curcumin, CAPE or resveratrol (10–40μM) prior to exposure to TNFα (1 or 10ng/ml) or FP-12 (20μM) for 2 hours. Nuclear protein extracts were purified from the cells (section 2.8) and analysed for NF-κB activity using EMSAs (section 2.9). A radioactive labelled oligonucleotide, containing a consensus NF-κB recognition motif, was incubated with equal amounts of nuclear extract before the complexes were separated onto a non-denaturing 4% polyacrylamide gel.

A very low-level of NF-κB DNA binding activity was detected in control cells (Figure 4.1A, lane 1), but treatment with TNFα at concentrations of 1 and 10ng/ml for 2 hours, caused a marked induction of nuclear protein binding to an oligonucleotide containing an NF-κB 'consensus' binding sequence, (Figure 4.1C, lane 2, and Figure 4.1B, lane 2 respectively). A 4-5 fold induction of NF-κB DNA binding to these oligonucleotides was observed, as seen by an increased intensity of the retarded band in cells exposed to TNFα compared to control cells, when measured with a phosphorimager (Molecular Dynamics). A similar induction of nuclear protein binding to an NF-κB oligonucleotide was observed with FP-12 (20μM), (Figure 4.1A, lane 2). In HCEC cells pretreated with
curcumin (10-40μM) for 1 hour prior to 10ng/ml TNFα, inhibition of NF-κB DNA binding activity was observed, but this was not dose dependent (Figure 4.1B, lanes 3-5). Pretreatment of HCEC cells with 10 and 20μM curcumin for 1 hour, prior to 1ng/ml TNFα for 2 hours, reduced NF-κB DNA binding activity, as observed by a decrease in band strength (Figure 4.1C lanes 3 and 4 respectively). The binding was completely inhibited by pretreatment with 40μM curcumin, (Figure 4.1C, lane 5). Curcumin (20μM) also reduced the NF-κB DNA binding activity in HCEC cells treated with FP-12 (20μM) by about 60%, (Figure 4.1A, lane 3).

To determine whether the inhibitory effects of curcumin on NF-κB DNA binding activity could be attributed to its antioxidant properties, the ability of CAPE, another phenolic antioxidant compound, structurally related to curcumin, to inhibit NF-κB DNA binding was investigated. CAPE (10-40μM) had little inhibitory effect on NF-κB DNA binding activity induced by 1ng/ml TNFα, (Figure 4.1C, lanes 6-8). Similar results were seen with FP-12, where CAPE had little inhibitory effect on NF-κB DNA binding activity (data not shown). Pretreatment of HCEC cells with resveratrol (10-40μM) for 1 hour prior to TNFα (1ng/ml) for 2 hours also, like CAPE, did not inhibit NF-κB DNA binding induced by TNFα, (Figure 1D, lanes 3-5), although a partial reduction in DNA binding was seen after pretreatment with 10μM resveratrol. Curcumin, CAPE and resveratrol alone (20μM) did not cause increased DNA binding of NF-κB, above that seen in untreated HCEC cells, (Figure 4.1C, lanes 9 and 10 and Figure 4.1D, lane 6, respectively).

Both TNFα and FP-12 strongly induced a slow migrating complex representing NF-κB DNA binding. Both untreated and stimulated cell nuclear extracts also produced an intense faster migrating DNA-protein complex. To determine the specificity of the binding to an NF-κB consensus sequence, binding was competed by incubation with a 200 fold molar excess of cold consensus NF-κB oligonucleotide, (Figure 4.1A, lane 5, Figure 4.1B, lane 7 and Figure 4.1C, lane 11), or 200 fold molar excess of an unrelated consensus oligonucleotide, AP-1 (Figure 4.1A, lane 6, Figure 4.1B, lane 8 and Figure 4.1C, lane 12). For the competition experiments, nuclear extracts were used from TNFα or FP-12 exposed HCEC cells. As shown by these competition studies with an excess of unlabelled 'cold' homologous oligonucleotide, the slower migrating complex represents
Curcumin 20 μM
Excess cold NF-κB
Excess cold AP-1
Figure 4.1: Effect of pretreatment with curcumin, CAPE or resveratrol on TNFα and FP-12 mediated NF-κB activation in HCEC cells. HCEC cells were pretreated with the indicated concentrations of curcumin (A, B and C), CAPE (C) or resveratrol (D) (10-40μM), for 1 hour followed by stimulation with TNFα (1 or 10 ng/ml) or FP-12 (20μM) for 2 hours. Nuclear extracts were prepared and analysed for NF-κB DNA binding activity by EMSA, with a 32P labelled consensus NF-κB probe. To determine specificity of the binding, nuclear extracts from FP-12 or TNFα exposed HCEC cells were incubated in the presence of a 200 fold molar excess of unlabelled NF-κB oligonucleotide, or a 200 fold molar excess of an oligonucleotide containing an AP-1 consensus site. In (D) only protein-DNA complexes are shown. The results show the gels from one experiment representative of three experiments.
specific binding to κB probes, since formation of the DNA-protein complex was inhibited by cold NF-κB oligonucleotide, but it was unaffected by an excess of cold AP-1 oligonucleotide. The faster migrating complex formed was inhibited by both AP-1 and NF-κB oligonucleotides indicating this was non-specific binding. The positions of the specific NF-κB binding complex and the free probe are indicated by arrows.

4.3: Determination of specificity of NF-κB DNA binding complexes

To determine which components of NF-κB were involved in the response to TNFα/FP-12 in HCEC cells supershift experiments were performed. The presence of the p65 subunit of NF-κB was tested since this is known to form transcriptionally active NF-κB complexes. Nuclear extracts from FP-12 (20μM) and TNFα (1ng/ml) exposed cells were incubated with an antibody to either the p65 (Rel A), subunit of NF-κB, a subunit contained in the transcriptionally active NF-κB-DNA binding complexes, or to an unrelated antibody, c-Fos, prior to analysis by EMSA. Antibodies to the p65 or p50 subunits of NF-κB can either inhibit DNA binding by masking the DNA binding domain, or induce a supershift. A supershift is induced when the antibody binds to a domain other than that essential for DNA binding and effectively increases the molecular weight of the NF-κB-oligonucleotide complex. Incubation with a polyclonal p65 antibody did not induce a supershift, although the band was shifted upwards in both TNFα (1ng/ml) and FP-12 (20μM) exposed cells, (Figure 4.2, lane 3 and lane 6 respectively). An unrelated antibody, against c-Fos, had no effect on the mobility of NF-κB, and the band appeared unaffected, when compared to an incubation without the antibody (Figure 4.2, lane 4 and lane 7, for TNFα and FP-12 exposed HCEC cells respectively). The lack of separation of the supershifted band from other non-p65 containing complexes can be explained by the fact that the gel was not run for long enough. The band observed is probably a combination of the 4 separate bands seen in the other EMSA gels (Figure 4.1), where the gels were run for a longer time, ie the 4 bands were not resolved on the gel in Figure 4.2.
Figure 4.2: Antibody supershift analysis of TNFα and FP-12 induced nuclear NF-κB DNA-protein complex. Nuclear extracts from unstimulated (lane 1), TNFα (1ng/ml), (lane 2) or FP-12 (20μM), (lane5) treated HCEC cells were subjected to EMSA using a 32P-labeled NF-κB probe. To determine the NF-κB species in the DNA-protein complex, a specific antibody recognising the p65 subunit of NF-κB (lanes 3 and 6) or an unrelated antibody to c-Fos (lanes 4 and 7) were incubated in the EMSA, along with the nuclear extracts from TNFα and FP-12 exposed HCEC cells. To determine the specificity of the binding, competition assays were performed with a 200 fold molar excess of 'cold' unlabeled NF-κB oligonucleotide, or a 200 fold molar excess of an oligonucleotide containing an AP-1 consensus site. The band representing NF-κB and the free, unbound probe, are indicated by arrows.
4.4: The effect of curcumin on NF-κB DNA binding to a COX-2 ‘promoter’ NF-κB oligonucleotide in cells exposed to TNFα

To determine if TNFα regulates COX-2 in HCEC cells through the NF-κB site in the promoter of this gene, the present studies have looked at the protein-DNA binding with an NF-κB oligonucleotide containing the NF-κB sequence found in the COX-2 promoter region nearest to the transcriptional start site, called the COX-2 ‘promoter’ NF-κB oligonucleotide (sections 2.10 and 2.11). Nuclear extracts from HCEC cells exposed to TNFα for 2 hours with or without pretreatment with 40pM curcumin for 1 hour were incubated with a 32P labelled ‘COX-2 promoter’ NF-κB oligonucleotide followed by analysis by EMSA. TNFα, 1ng/ml, induced binding to the COX-2 promoter NF-κB oligonucleotide, (Figure 4.3, lane 3). Complex formation of some of the bands in the mutated version of this oligonucleotide were reduced indicating that the binding was sequence specific, (Figure 4.3, lane 4). Since the lower two bands were similar in intensity in both the wild type and mutated oligonucleotide, further work is required to determine which of the bands contain the NF-κB p65 subunit. Pretreatment of HCEC cells with 40pM curcumin, prior to 1ng/ml TNFα, greatly inhibited NF-κB DNA binding to the wild type COX-2 promoter NF-κB oligonucleotide, to a level that was observed in unstimulated cells, (Figure 4.3, lane 5). Curcumin alone (40μM) had no effect on NF-κB DNA binding activity, (Figure 4.3, lane 7).

4.5: Effect of FP-12 on AP-1 DNA binding in EMSAs

To determine whether FP-12 specifically induces the activation of NF-κB, or also affects other transcription factors, the effect of FP-12 on the induction of the transcription factor complex AP-1, another oxidant stress sensitive transcription factor was investigated in HCEC cells. Nuclear extracts from control and FP-12 treated cells were incubated with a 32P labelled DNA probe which allows for the detection of the DNA-binding activities of AP-1. Specificity of the protein-DNA complex was tested by competition with the respective unlabelled oligonucleotide. FP-12 (5 and 20μM) had no marked effect on AP-1 DNA binding activity, (Figure 4.4, lanes 4 and 5). Even though the HCEC cells were serum starved in 2% FCS for 24 hours prior to treatment with FP-12, there was quite a high constitutive level of AP-1 DNA binding.
Figure 4.3: Effect of curcumin on NF-κB DNA binding to a COX-2 promoter NF-κB oligonucleotide, in HCEC cells exposed to TNFα. HCEC cells were exposed to TNFα (1ng/ml) for 2 hours with and without pretreatment with curcumin, 40μM, for 1 hour. Nuclear extracts were prepared from the HCEC cells and incubated with a 32p-labelled COX-2 promoter oligonucleotide, containing the sequence for NF-κB found in the COX-2 gene promoter region, nearest to the transcriptional start site (WT). The nuclear extracts were also incubated with a mutated form of this oligonucleotide (M), to determine if the binding was sequence specific. The results show the gel from one experiment representative of two experiments.
Figure 4.4: Effect of FP-12 on AP-1 DNA binding activity. HCEC cells were exposed to TPA (100ng/ml), deoxycholic acid (50μM) or FP-12 (5 and 20μM), for 2 hours. Nuclear proteins were extracted from the HCEC cells and the extracts were incubated with a 32P-labelled oligonucleotide probe containing the consensus AP-1 DNA binding site. Protein-DNA complexes formed were fractionated on a 4% polyacrylamide gel. Binding competition assays were performed with a 200 fold molar excess of ‘cold’ AP-1 oligonucleotide (lane 6), to compete for the AP-1 complex, or with a 200 fold excess of a ‘cold’ unrelated NF-kB oligonucleotide (lane 7), to determine the specificity of the complexes formed. Only protein-DNA complexes are shown. The specific AP-1 band is shown by an arrow, with the lower band being a non-specific band. The gel shown is a representative gel, from two separate experiments performed on different cell cultures on different occasions.
activity present in unstimulated cells, (Figure 4.4, lane 1). TPA (100ng/ml), a known activator of AP-1 in many cell types, only increased the DNA binding of AP-1 by about 2 fold above that seen in unstimulated HCEC cells, (Figure 4.4, lane 2). He bile acid, deoxycholic acid (50μM) also failed to increase AP-1 DNA binding activity above that observed in unstimulated cells.

4.6: Effect of H2O2 on the DNA binding activity of NF-κB in the presence and absence of various agents

To check the possibility that oxygen radicals are involved in the activation of NF-κB in HCEC cells, the effect of H2O2 on NF-κB DNA binding activity was investigated. H2O2 can activate NF-κB by producing reactive oxygen intermediates (Schreck et al, 1991) that can activate a signalling pathway leading to phosphorylation of IκB and release of NF-κB. H2O2 is a membrane permeable agent which allows studies of the effect of ROS in living cells. Nuclear extracts were prepared from cells (section 2.8) treated with 1mM H2O2 for 2 hours, with or without pretreatment for 1 hour with 0.1 and 1mM sodium salicylate, 40μM curcumin or 40μM CAPE. Equal amounts of the nuclear extracts were incubated with a 32P labelled oligonucleotide encompassing a NF-κB consensus motif, followed by analysis by EMSAs (section 2.9). H2O2 caused a marked induction of NF-κB DNA binding activity, (Figure 4.5, lane 2). Sodium salicylate caused a dose dependent inhibitory effect on the NF-κB DNA binding activity induced by H2O2, where DNA binding was reduced to nearly control levels, in HCEC cells pretreated with 1mM sodium salicylate, (Figure 4.5, lanes 5 and 6). Curcumin (40μM) completely inhibited the NF-κB DNA binding activity induced by H2O2, (Figure 4.5, lane 3). The same concentration of CAPE (40μM), however showed little or no inhibitory effect on the NF-κB DNA binding activity induced by H2O2, (Figure 4.5, lane 4). Exposure of HCEC cells to 1mM sodium salicylate, 40μM curcumin or 40μM CAPE alone, had no effect on NF-κB DNA binding activities, (Figure 4.5, lanes 7- 9 respectively).
Figure 4.5: Effect of H$_2$O$_2$ treatment, in the presence and absence of curcumin, CAPE and sodium salicylate, on NF-κB DNA-binding activity in HCEC cells. HCEC cells were exposed to H$_2$O$_2$ (1mM), for 2 hours, without pretreatment (lane 2) or with pretreatment for 1 hour with curcumin (40μM) (lane 3), CAPE (40μM) (lane 4) or sodium salicylate (0.1 and 1mM) (lanes 5 and 6, respectively). Nuclear extracts were prepared from the cells and analysed for NF-κB DNA binding activity by EMSA, with a $^{32}$P labelled consensus NF-κB probe. The band representing NF-κB is indicated by an arrowhead, and the lower arrowhead indicates the position of unbound DNA. The gel shown is a representative gel, from two experiments performed on different cell cultures on two different occasions.
4.7: Effect of curcumin on TNFα-induced NF-κB dependent transactivation

To determine if the activation of NF-κB by TNFα relates to an increase in transactivation of genes containing κB binding sites, by this transcription factor, transient transfection assays were performed, by Rebecca Munks, (MRC Toxicology Unit, Leicester University, UK) (section 2.12). An NF-κB reporter construct linked to a luciferase reporter containing 6 NF-κB DNA consensus sequences (p6-NF-κB) was used for transfections. HCEC cells could not be used for the transfection experiments because they exhibited poor transfection efficiency and the electroporation procedure was toxic to the cells. Instead a colon carcinoma cell line (SW480), with low constitutive COX-2 levels, was used. An 'empty cassette’, lacking the NF-κB binding sequences was used as a negative control. Following transfection of the constructs by electroporation, the cells were treated with 10ng/ml TNFα for 4 hours with or without pretreatment for 1 hour with 20μM curcumin. The transcriptional activation of the construct was monitored by measuring luciferase activity.

SW480 cells transfected with the ‘empty cassette’ and exposed to 10ng/ml TNFα, showed no difference in luciferase activity compared to untreated cells. However exposure of cells, transiently transfected with p6NFκB (and a β galactosidase plasmid (pCMVgal), to normalise for differences in transfection efficiency), to TNFα (10ng/ml), caused a threefold induction of p6NFκB luciferase activity, (Figure 4.6). Pretreatment for 1 hour with 20μM curcumin inhibited the activation of p6NFκB mediated luciferase activity, by TNFα, indicating that curcumin inhibits the transactivating potential of NF-κB in these cells (Figure 4.6).

4.8: Effect of curcumin on TNFα induced IκB degradation

The translocation of NF-κB complexes to the nucleus and transactivation of NF-κB dependent gene expression, is preceded by the phosphorylation and subsequent degradation of IκBα, the NF-κB sequestering protein. To determine whether the inhibitory action of curcumin on NF-κB DNA binding was due to its effect on IκBα degradation, protein levels of IκBα were examined by Western blot analysis with an
Figure 4.6: Effect of curcumin on TNFα induced NF-κB dependent transactivation. Transient transfection assays were performed with SW480 cells, using a construct containing 6 NF-κB binding DNA consensus sequences and a luciferase reporter gene (p6-NF-κB-tk-luc), and with the control plasmid tk-36-luc (an ‘empty cassette’). 24 hours after transfection, the SW480 cells were left untreated or exposed to TNFα (10ng/ml), for 4 hours, with or without pretreatment for 1 hour with curcumin (20μM), prior to measuring luciferase reporter gene activity.

Luciferase activity units were normalised to β-galactosidase activity, and the results expressed as percentage expression relative to untreated cells. The values represent the means +/- SD of three separate transfections carried out in duplicate. Statistical significance was determined (* p < 0.01) by analysis of variance (students t test) where cells treated with TNFα differ significantly from untreated cells.
anti-IkBα IgG specific antibody (section 2.15). Figure 4.7 shows the time course of degradation of IkBα in cells treated with TNFα or FP-12. A band representing IkBα was observed in unstimulated cells, (Figure 4.7, lane 1). HCEC cells were exposed to 20μM FP-12 for various times between 15 minutes to 3 hours. Degradation of IkBα was not observed after exposure to FP-12 for the times indicated, (Figure 4.7A(1), lanes 2-6). After exposure to FP-12 for shorter periods of time, from 5 minutes to 1 hour, no degradation of IkBα was observed, and a band representing IkB persisted as seen in unstimulated cells, (Figure 4.7B(1)).

When HCEC cells were exposed to TNFα, IkBα was degraded after exposure for 15 minutes, (Figure 4.7A, lane 2), but after exposure to TNFα for 2 hours a band was seen similar to that observed in unstimulated cells, suggesting that IkBα was resynthesised. HCEC cells were exposed to TNFα for shorter periods of times between 5 minutes to 1 hour, prior to analysis for IkBα. After exposure to TNFα for 10 minutes the IkB band had completely disappeared indicating degradation. However, this was followed by a period of slow recovery, and the band for IkBα reappeared as seen in unstimulated HCEC cells, after 1 hour exposure to TNFα (Figure 4.7B (2) lane 6).

To determine if curcumin could prevent the degradation of IkBα by TNFα, HCEC cells were pretreated with 40μM curcumin for 1 hour prior to TNFα (10ng/ml). Curcumin pretreatment completely protected IkBα from proteolysis by the 26S proteasome as indicated by the presence of a band, after exposure to TNFα for 10 minutes, (Figure 4.7B(3), lane 3). Degradation of IkBα was not observed in cells pretreated with curcumin and a band representing IkBα persisted, throughout the 1 hour exposure to TNFα, (Figure 4.7B(3), lanes 3-6). In contrast, pretreatment of HCEC cells with 100μM resveratrol, for 1 hour prior to TNFα for the indicated time points, had no inhibitory effect on the degradation of IkBα induced by TNFα, (Figure 4.7B(4)). A second signal, with slightly slower migration was observed, probably representing the phosphorylated form of IkBα, after 5 minutes exposure to TNFα, (indicated by an asterisk) (Figure 4.7B (4), lane 2). This was followed by the degradation and reappearance of IkBα over the same time course as exposure to TNFα alone.
Figure 4.7: Time course analysis of IκBα degradation by FP-12 and TNFα. HCEC cells were either untreated or incubated with TNFα (10ng/ml) or FP-12 (20μM) for the indicated time periods, with or without pretreatment for 1 hour with curcumin (40μM) or resveratrol (100μM). Complete protein extracts were isolated from the HCEC cells, and analysed by Western blots, with an IκBα specific antibody. An asterisk represents the phosphorylated form of IκBα. No other protein complexes were detected except those shown. The results shown are a representative experiment from three separate experiments performed on different cell cultures on different occasions.
4.9: Effect of FP-12 on PKC activation

The signal transduction pathway involved in the activation of NF-κB by TNFα apparently does not require PKC (Meichle et al, 1990). However it is possible that FP-12 could activate PKC which can directly phosphorylate IκB to release the inhibitor and allow translocation of NF-κB to the nucleus. In unstimulated cells PKC remains loosely attached to the cytoplasmic side of the plasma membrane. In an inactive state the enzyme is readily recovered as a cytoplasmic protein. Upon activation by various stimuli, such as phorbol esters and bile acids (Zhang et al, 1998), the enzyme associates with the membrane and can be recovered in the membrane or particulate fraction of the cell.

To determine if PKC is involved in the activation of NF-κB by FP-12, HCEC cells were exposed to 100ng/ml TPA or 5 and 20μM FP-12, for 1 hour, and nuclear, membrane and cytoplasmic proteins were extracted from the cells. Western blots were performed with antibodies specific for PKCα and PKC zeta. In unstimulated HCEC cells, PKCα was located only in the cytosolic fraction of the cells, (Figure 4.8A, lane 1). TPA (100ng/ml) translocated PKCα from the cytosolic to the membrane and nuclear fractions of the cells, (Figure 4.8A, lanes 2, 6 and 10). A band was not detected in the cytosolic fraction, after exposure to TPA, but an intense band in the membrane fraction, and a slight band in the nuclear fraction was seen. Treatment of HCEC cells with FP-12 (5 and 20μM), caused a slight reduction (~35%) in PKCα levels detected in the cytosolic fraction, compared to unstimulated cells, (Figure 4.8A, lanes 3 and 4). PKCα was also observed (faint bands), in the membrane, (Figure 4.8A, lanes 7 and 8), and nuclear fractions, (Figure 4.8A, lanes 11 and 12), of the HCEC cell protein extracts from cells exposed to 5μM and 20μM FP-12 respectively. This observation suggests that FP-12 caused some activation of PKCα, as seen by a slight translocation from the cytosol to the membrane and nuclear protein fractions, upon FP-12 treatment.

It appears that in HCEC cells PKC zeta exists in 3 phosphorylation states, as suggested from the 3 bands observed, representing PKC zeta, (Figure 4.8B). In unstimulated HCEC cells PKC zeta was detected in the cytosolic fraction of the cells, (Figure 4.8B, lane 1, top band). In HCEC cells exposed to TPA (100ng/ml) only a low level of PKC zeta remained in the cytosolic protein fraction, (Figure 4.8B, lane 2), whereas strong
Figure 4.8: Effect of FP-12 on PKC activation. HCEC cells were either unstimulated or exposed to FP-12 (5 and 20μM) or TPA (100ng/ml) for 1 hour, and the nuclear, cytoplasmic and membrane protein was extracted from the cells. Western blot analysis was performed with PKCα (A) or PKC zeta (B) specific antibodies. No other protein complexes were detected except those shown. The results shown are a representative gel, from 2-3 separate experiments performed on different cell cultures on different occasions.
bands were detected in both the membrane, (lane 6) and nuclear, (lane 10) protein fractions. Exposure of HCEC cells to FP-12 (5 and 20µM) had little effect on the location of PKC zeta where it remained predominantly in the cytosolic fraction of the cells, (Figure 4.8B, lanes 3 and 4). However, more intense bands than those seen in unstimulated HCEC cells, were detected in both the membrane (Figure 4.8B, lanes 7 and 8) and nuclear protein fractions (Figure 4.8B, lanes 11 and 12), after exposure to FP-12, indicating that FP-12 caused a slight ‘activation’ of PKC zeta.

4.10: Discussion

In the cell NF-κB is stored in the cytoplasm in an inactive state by its interaction with IκBα. On activation, IκB undergoes degradation through a ubiquitin dependent pathway (Sun et al, 1993) allowing translocation of NF-κB to the nucleus and subsequently binding to DNA regulatory elements within NF-κB target genes. The results show that in HCEC cells TNFα caused a rapid degradation of IκB after exposure for 5 minutes, but FP-12 did not cause IκB to degrade over a 3 hour exposure time. However both TNFα (1 and 10ng/ml) and FP-12 (20µM) increased NF-κB DNA binding by about four to five-fold above that seen in control HCEC cells and FP-12 increased NF-κB DNA binding complexes with profiles in EMSAs similar to TNFα. Therefore the TNFα induced degradation of IκB-α, is an event that is associated with the increased formation of NF-κB complexes. Since resynthesis of IκBα occurred after 1 hour exposure to TNFα, as seen by the reappearance of IκB by Western blots, and activation of NF-κB occurred after 2 hours exposure to TNFα, this could suggest that the degradation of IκB-β by TNFα could account for the prolonged activation of NF-κB (Thompson et al, 1995). Johnson et al (1996) proposed that IκBβ may contribute to the persistent NF-κB activation by TNFα in HUVEC cells. However the effects of TNFα on IκB-β degradation was not investigated in this study. The activation of NF-κB by TNFα in this study was expected, since Barnes and Karin (1997), reported that the principal transcription factor induced by TNFα is NF-κB.

It has always been reported that the mechanism of NF-κB activation involves degradation of IκB. Giri and Aggarwal (1998) reported that degradation of IκBα is critical for NF-κB activation and translocation to the nucleus. However they also
reported that Hut-78 cells constitutively express high levels of IkB-α along with activated NF-κB. Recently an alternative mechanism of NF-κB activation has been reported to occur in Jurkat T cells stimulated with pervanadate. In this case tyrosine phosphorylation of IkBα activates NF-κB without proteolytic degradation of IkBα (Imbert et al, 1996). Janssen-Heininger et al (1999) also showed that H₂O₂ can transactivate NF-κB dependent gene expression without causing IkB degradation in rat lung epithelial cells. When HCEC cells were exposed to FP-12, it was possible that IkB was resistant to proteolysis or the protease was not active. Also it was possible that when HCEC cells were exposed to FP-12, MEKK1 was activated which is capable of signalling through the IkB/NF-κB system without causing IkB degradation. Jobin and Sartor (1998) suggested that a defect in IkB degradation may result from low IkB kinase activity, high phosphatase activity or defective proteosome function. My results suggest that the mechanisms of NF-κB activation by cytokines such as TNFα, and agents such as FP-12, is different.

Pretreatment of HCEC cells with curcumin for 1 hour prior to TNFα caused a dose dependent inhibition of NF-κB DNA binding activity; pretreatment with 40μM curcumin almost completely inhibited the activation of NF-κB by TNFα. This is consistent with the reports of Kumar et al (1998) who observed that after 1 hour pretreatment with curcumin, prior to TNFα (0.1nM for 30 minutes) a dose dependent inhibition of NF-κB activation was observed, with an approximate 90% suppression occurring after pretreatment with 40μM curcumin. Curcumin has been shown to inhibit the activation of NF-κB DNA binding in bone marrow stromal cells by both IL-1α and TNFα (Xu et al, 1997). The EMSA of nuclear extracts from cells exposed to TNFα for 2 hours, after pretreating with curcumin for 1 hour, and the Western blot results of the effect of curcumin on TNFα induced IkB degradation reveals that curcumin suppresses the degradation of IkB and this serves to prevent the release of NF-κB. This is similar to what was reported by Singh and Aggarwal, (1995), who showed that curcumin prevented NF-κB activation by inhibiting the TNFα dependent degradation of IkB in ML-1A cells, a human myelomonoblastic leukemia cell line. Brennan and O’Neil (1998) observed that treatment of Jurkat cells with TNFα (10ng/ml) for 20 minutes, caused a marked degradation of IkBα, which was inhibited by pretreatment with 150μM curcumin. The observation that curcumin inhibited the degradation of IkB
induced by TNFα indicates that the step in the signal transduction pathway of NF-κB activation inhibited by this agent is at or before the phosphorylation of IκB (Kumar et al, 1998). Chen and Tan (1998) reported that curcumin can inhibit JNK, a down-stream kinase of MEKK1, which can also cause inhibition of NF-κB activation. Curcumin could also have inhibited IκB degradation by either limiting access of the NF-κB-IκB complex to the proteosome or by blocking proteosome activity directly. As has been shown with other inhibitors, the results in the present study suggest that the effect of curcumin was not due to the chemical modification of NF-κB proteins (Kumar et al, 1992), because the inhibitory effects of curcumin were seen without interfering with NF-κB binding directly. It has to be noted that the time of curcumin administration to cells is important, since Bierhaus et al (1997) who looked at the effect of curcumin on TNFα mediated NF-κB activation in Bovine aortic endothelial cells saw that pretreatment with curcumin for 1 hour before stimulation with TNFα reduced NF-κB activation whereas exposure to curcumin 15 minutes after TNFα had no inhibitory effect. This pattern was also observed by Singh and Aggarwal (1995) who observed that the TNFα response was only inhibited when cells were pretreated with curcumin, with no inhibition of NF-κB activation noted when cells were co-incubated with TNFα and curcumin, or curcumin was given after TNFα administration. This indicates that it is not only the dose of the chemopreventive agent that is important but also the time of administration, to effectively inhibit NF-κB activation.

The present study also examined whether NF-κB could bind to an oligonucleotide containing an NF-κB consensus sequence found in the COX-2 promoter. TNFα (10ng/ml) induced NF-κB DNA binding to the COX-2 promoter NF-κB oligonucleotide, suggesting that this transcription factor may be involved in the transactivation of the COX-2 gene. Since curcumin inhibited this TNFα mediated binding of NF-κB it is possible that the inhibitory effects of curcumin on COX-2 expression are mediated at least in part through inhibition of this transcription factor. Although TNFα induced binding to part of the COX-2 promoter containing the 3’ NF-κB site, the binding was reduced when using an NF-κB oligonucleotide in which the sequence was mutated. The results show that TNFα not only induces binding of nuclear protein to a consensus NF-κB oligonucleotide, but also to the NF-κB consensus sequence that is present in the promoter region of the COX-2 gene closest to the
transcriptional start site. A wild type NF-κB oligonucleotide showed increased DNA binding of NF-κB following TNFα exposure, in EMSAs, but the mutant form was ineffective at inducing DNA binding activity. This data suggests that TNFα may stimulate COX-2 expression in HCEC cells by signalling via NF-κB and that inhibition of COX-2 expression by curcumin may be via inhibition of this binding.

To determine if the inhibitory effects of curcumin on NF-κB DNA binding was due to an antioxidant effect of the agent, the effect of CAPE, an antioxidant structurally related to curcumin, on NF-κB DNA binding activity was investigated. Since Natarajan et al (1996) found TNFα responses were only inhibited when cells were pretreated with CAPE, HCEC cells were pretreated for 1 hour with CAPE, prior to TNFα. CAPE showed no inhibitory effect on NF-κB activation induced by TNFα. This could be because CAPE exerts its effect in the nucleus by impairing the transcriptional activity of NF-κB already bound to DNA, ie only influences the binding of activated NF-κB in vitro. Natarajan et al (1996) showed that a higher concentration of CAPE than has been used in the present study, inhibited NF-κB activation in U937 cells, not by blocking the degradation of IkBα, but by suppressing the interaction of NF-κB proteins with the DNA directly. CAPE could directly inhibit the DNA binding of activated NF-κB by modifying reactive amino acids such as cysteine residues in the DNA binding or dimerisation domains. Therefore if nuclear protein extracted from TNFα exposed HCEC cells, with nuclear NF-κB, were incubated with various concentrations of CAPE in vitro, prior to the addition of the NF-κB oligonucleotide and looking for DNA binding activity of NF-κB by EMSAs, inhibition of DNA binding may have occurred. This illustrates that the effects on factor binding to DNA regulatory sequences (and therefore gene expression) by phenolic antioxidants are highly complex, when the effects of curcumin are compared to CAPE. For example curcumin and CAPE can both inhibit NF-κB activation; curcumin prevented translocation of the p65 subunit of NF-κB from the cytoplasm to the nucleus by inhibiting IkB degradation whereas CAPE mediated inhibition is reported to be affected at the level of binding of NF-κB to DNA.

Resveratrol pretreatment, in agreement with observations by others using CAPE (Natarajan et al, 1996) did not inhibit IkB degradation in TNFα treated HCEC cells. After exposure to TNFα for 5 minutes, subsequent to the pretreatment with resveratrol,
two bands were detected, with a slower migrating band representing the phosphorylated form of IκB. This has often been observed in cells exposed to TNFα and suggests that TNFα caused a rapid phosphorylation of IκBα followed almost immediately by its degradation. This may explain why pretreatment of HCEC cells with 40μM resveratrol prior to TNFα, did not inhibit TNFα induced NF-κB activation. It could be speculated that resveratrol, like CAPE, does not inhibit NF-κB activation by blocking the degradation of IκB, but may only have inhibitory activity, if incubated in vitro, to suppress the interaction of NF-κB proteins with the DNA.

One inhibitor of NF-κB activation is the cysteine derivative and glutathione precursor NAC, a classical antioxidant. However no inhibitory effect of NAC on NF-κB DNA binding activity was observed in HCEC cells exposed to FP-12 or TNFα. Meyer et al (1993) reported that high concentrations of NAC in the millimolar range are required to observe inhibitory effects on NF-κB activation. Therefore a higher concentration of NAC may have inhibited NF-κB activation in these studies. Since curcumin could inhibit NF-κB activation, whereas NAC had no inhibitory activity, this suggests that the ability of curcumin to inhibit activation and translocation of NF-κB to the nucleus was unrelated to its antioxidant properties. Brennan and O’Neill (1998) have also suggested that the inhibitory effect of curcumin on NF-κB activation was not just due to its antioxidant properties. They observed that curcumin could inhibit NF-κB DNA binding in vitro, when incubated with nuclear extracts from TNFα stimulated cells suggesting that curcumin can interfere with DNA binding directly.

The results of the super-shift experiment to determine the protein subunits present in the NF-κB complex, are difficult to interpret because the gel was not run for long enough, so the NF-κB complex did not resolve into four bands as seen in the other EMSA gels. However in both the TNFα and FP-12 treated cells the band size was broader when the protein-DNA complex was incubated with an antibody to p65 than that seen without incubation with a p65 antibody. This slight supershift suggests that the complex did contain p65. The other component in this band may be p50, given the widespread occurrence of NF-κB as a p65:p50 heterodimer and the next band could be a p50:p50 homodimer, but this remains to be determined, in the present study. Gallois et al (1998) have shown that in human hepatic stellate cells, TNFα induced a slow migrating band
containing p65 and p50, and p50 in the faster migrating complex. Dimeric complexes of NF-κB that contain p65 function as strong activators of gene expression (Siebenlist et al, 1994). A p50:p50 homodimer binds more avidly to consensus DNA binding sites than a p50:p65 heterodimer, but is not an effective inducer of transcription. Therefore p50:p50 homodimer formation and binding inhibits the effect of p50:p65 function by competing with their binding to DNA.

Results of the present studies show that FP-12 increased the binding of NF-κB, suggesting that this transcription factor may be a key mediator in the cellular response after exposure to FP-12. Numerous studies have pointed to a role of reactive oxygen intermediates as a common second messenger system used by different stimuli to activate transcription factors. AP-1 and NF-κB were the first eukaryotic transcription factors shown to respond directly to oxidative stress (Sen and Packer, 1996). Therefore many agents that activate NF-κB stimulate the production of ROS (Schreck et al, 1991). FP-12 could act as a tumour promoter by producing ROS in cells. Plummer and Faux (1994) have previously shown that FP-12 can induce oxidative stress in cells. The effect of FP-12 on the binding activity of AP-1 was also examined, since as mentioned above, AP-1 is another transcription factor thought to alter gene expression in response to many different agents, regulated by redox mechanisms. However FP-12 did not have a marked effect on the DNA binding of AP-1, in HCEC cells, compared to unexposed cells. This shows that activation of NF-κB by FP-12 was specific, and was not necessarily due to the induction of an intracellular oxidative stress by FP-12.

Since, as mentioned above, some studies have demonstrated that in particular activation of NF-κB requires oxidative signalling, ie its expression is dependent on the redox state of the cell, this study looked at the effect of H₂O₂ with and without pretreatment with curcumin, on NF-κB activation. The finding that H₂O₂ induces NF-κB activation in HCEC cells after two hours is similar to the results of Schreck et al (1991) who observed maximal induction of NF-κB by H₂O₂ after 2-4 hours exposure. Like TNFα, NFκB activation by H₂O₂ involves phosphorylation of IκB at serines 32 and 36, which is necessary for rapid degradation of the protein by the proteosome. Curcumin could be preventing IκB degradation and therefore inhibiting H₂O₂ induced NF-κB activation. However since the signal transduction pathways induced by TNFα and H₂O₂, leading to NF-κB activation differ, and curcumin can inhibit NF-κB activation by both agents, the
data suggests that curcumin may inhibit NF-κB activation via inhibiting at a step where the two signalling pathways converge. Unlike curcumin which completely inhibited NF-κB DNA binding induced by H₂O₂, CAPE showed little inhibitory effect at an equimolar concentration (40μM) to curcumin. Sodium salicylate (0.1mM) caused a slight inhibition of binding, but NF-κB DNA binding activity was almost completely inhibited by pretreatment with 1mM sodium salicylate.

Since curcumin can inhibit signalling via PKC (Liu et al, 1993), and PKC is known to activate NF-κB, by phosphorylating and degrading IκB, releasing NF-κB (Ghosh and Baltimore, 1990), it is possible that PKC could be an important signalling molecule for the observed activation of NF-κB by FP-12. Inhibition of PKC activation by curcumin could block the effects of FP-12 on NF-κB activation and COX-2 gene expression. It has previously been shown that FP-12 can act as a cofactor for PKC (Hoshina et al, 1991) and bile acids are known colon tumour promoters that activate PKC, which may be their mechanism of inducing carcinogenesis (Zhang et al, 1998). FP-12 did cause some activation of PKC but was not as marked as the effect of TPA, an agent that is known to regulate gene expression by activating signal transduction pathways involving PKC (Karin, 1995). The PKC isoforms PKCα and PKC zeta were chosen to examine the effect of FP-12 on the subcellular location of this enzyme, because these isoforms have been found to be present in the cytosol of unstimulated normal colonic epithelial cells (Kahl-Rainer et al, 1994). The results of the present study suggests that FP-12 may activate NF-κB in part, by a signalling pathway dependent on PKC. However, since the activation of PKC by FP-12 was only slight this may only contribute part of the signalling events due to FP-12, leading to NF-κB activation. Activation of NF-κB DNA binding by TNFα appears to be independent of PKC (Meichle et al, 1990). Although TNFα can induce a rapid and transient activation of PKC, depletion of PKC by PKC inhibitors does not affect NF-κB activation by TNFα. The signalling cascade activated upstream of IκB-α has been recently characterised and involves a complex of kinases, NIK, and two isoforms of IkappaB kinase, IKKα and IKKβ (DiDonato et al, 1997). However, the signalling pathway that leads to the activation of NF-κB may differ from one cell type to another, and among inducers within a cell type.
To determine whether the NF-κB elements in the COX-2 promoter were responsible for transcriptional activation of COX-2 by TNFα, transient transfection assays, with a plasmid containing 6 NF-κB sequences linked to a luciferase reporter construct, were performed. The observation in the present study showing the transcriptional activation of NF-κB dependent gene expression by TNFα, are consistent with the induction of DNA binding of NF-κB by this agent. The results show that treatment of HCEC cells with TNFα, not only induced the specific binding of an NF-κB sequence present in the COX-2 promoter, but also induced transactivation of NF-κB dependent genes. This indicates that NF-κB is partly involved in the activation of COX-2 gene expression by the tumour promoter TNFα and its inhibition by curcumin.
CHAPTER 5: CONSEQUENCES OF NF-κB INHIBITION
BY CURCUMIN ON CELL DEATH
5.1: Introduction

The growth of a tumour is regulated by two mechanisms; by the rate at which the tumour cells proliferate and by the rate at which these cells die, an event which is largely dependent on apoptosis (Kerr et al, 1994). Apoptosis plays an essential role as a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells, or excess cells that have been improperly induced to divide by a mitotic stimulus. Recent evidence suggests that the failure of cells to undergo apoptotic cell death might be one possible mechanism of tumour promotion (Wright et al, 1994), leading to excessively proliferating cells, contributing to tumour growth and the promotion of neoplastic progression (Payne et al, 1995; Bedi et al, 1995). Apoptosis is morphologically characterised by decreased cell and nuclear volume and condensation of chromatin during later phases of the apoptotic process. At very late stages of apoptosis cell fragments are ultimately phagocytosed by other cells or macrophages. Because cell death is an active process of gene directed cellular self destruction, the molecular signals which control this process leading to apoptosis could be important targets in chemoprevention and therapy, to modulate the growth of a tumour.

It has recently been shown that the resistance to TNFα induced cell death results from the ability of TNFα to activate NF-κB-mediated transcription (Van Antwerp et al, 1996). Therefore activation of NF-κB and its downstream factors are likely to play a major role in cell survival. It has been shown previously that increased expression of COX-2 in rat intestinal epithelial cells caused the cells to adhere more to the extracellular matrix and made them resistant to apoptosis (Tsujii and DuBois, 1995).

Increasing interest has focused on new antitumour strategies which involve regulation of the cell death program. One mechanism of action of chemopreventive agents is to induce apoptosis, and this property explains at least in part their actions (Elder et al, 1996). NSAIDs are capable of inducing apoptosis, and NSAID treatment decreases the rate of growth of many intestinal and non-intestinal tumour cell lines (Shiff et al, 1995; Schiff et al, 1996). Pasricha et al (1995) have observed a significant increase in apoptosis in the colonic mucosa of FAP patients after treatment with the NSAID,
sulindac. Boolbol et al (1996) found that the induction of apoptosis by sulindac is associated with cyclooxygenase inhibition.

Hanif et al (1997) reported that curcumin can inhibit cell proliferation. Jiang et al (1996b) observed that curcumin could stop the growth of the human colon cancer cell line HT29, with various common features of apoptosis. It is possible that pretreating cells with curcumin prior to TNFα or FP-12 could trigger apoptosis by the complete or near complete inhibition of the expression of COX-2, via inhibition of NF-κB activation.

5.2: Effect of exposure of HCEC cells to TNFα or FP-12 in the presence or absence of curcumin on cell viability

5.2.1: Trypan Blue Exclusion assay

To assess the effect of curcumin on cell viability, HCEC cells were pre-treated for 1 hour with 10, 20 or 40 μM curcumin, prior to exposure to 10ng/ml TNFα or 20μM FP-12 for 6 hours. Aliquots of cells were counted using a haemocytometer and tested for viability by the trypan blue dye exclusion method (section 2.4.1). Cells that stained blue were considered dead (non-viable). At least 500 individual cells, from a minimum of 4 different fields were counted, for each sample.

Exposure of HCEC cells to TNFα or FP-12 alone had no effect on cell viability, and the cells were almost 100% viable, (Table 5.1). Exposure to curcumin (40μM) alone also caused no marked reduction in cell viability, the viability of the cells was still 98.3% (+/- 1.2%). Pretreatment of HCEC cells with 10μM curcumin prior to 10ng/ml TNFα had no effect on the viability of the cells. However pretreatment with 20μM curcumin, for 1 hour, prior to TNFα, for 6 hours caused a marked decrease in viable cells, as noted by the levels of trypan-blue positive cells, down to only 26.7 (+/-2.0)% viable cells, and pretreatment with 40μM curcumin decreased the cell viability even further, down to 4.4 (+/-1.7)% viable cells. Pretreatment of HCEC cells with 20μM curcumin prior to 20μM FP-12 had no marked effect on cell viability, cell viability equalling 98.2 (+/-1.8)%.
### Table 5.1: Effect of curcumin pretreatment on HCEC cell viability

HCEC cells were exposed to curcumin for 1 hour prior to TNFα or FP-12, for 6 hours. Cells were counted with a haemocytometer and viability determined by the trypan blue exclusion method. The results shown are the percentage viable cells after each treatment and are the mean +/- SE of 4 separate determinations.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>% VIABLE CELLS</th>
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<tbody>
<tr>
<td>Control (untreated)</td>
<td>99.5 (+/-0.6)</td>
</tr>
<tr>
<td>Curcumin (40μM)</td>
<td>98.3 (+/-1.2)</td>
</tr>
<tr>
<td>TNFα (10ng/ml)</td>
<td>99.1 (+/-0.6)</td>
</tr>
<tr>
<td>FP-12 (20μM)</td>
<td>98.9 (+/-0.5)</td>
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<tr>
<td>TNFα (10ng/ml) + Curcumin (10μM)</td>
<td>99.3 (+/-1.0)</td>
</tr>
<tr>
<td>TNFα (10ng/ml) + Curcumin (20μM)</td>
<td>26.7 (+/-2.0)</td>
</tr>
<tr>
<td>TNF (10ng/ml) + Curcumin (40μM)</td>
<td>4.4 (+/-1.7)</td>
</tr>
<tr>
<td>FP-12 (20μM) + Curcumin (20μM)</td>
<td>98.2 (+/-1.9)</td>
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5.2.2: MTT assay

Another assay to further assess the cytotoxicity of curcumin in HCEC cells, is the MTT assay. HCEC cells were pretreated for 1 hour with 10, 20 or 40μM curcumin or resveratrol prior to treatment with 10ng/ml TNFα or 20μM FP-12, for 24 hours. After exposure the viability of the cells was measured by the MTT assay (section 2.4.2). The viability of the treated cells was determined as a percentage of the untreated cells.

Exposure of HCEC cells to 10ng/ml TNFα and 20μM FP-12, alone, did not cause a reduction in cell viability (Figure 5.1). Pretreatment for 1 hour with 10μM curcumin prior to TNFα or FP-12 for 24 hours had no marked effect on cell viability (Figure 5.1A). However, pretreatment with 20μM curcumin prior to TNFα decreased the viability of the cells down to 84.1 +/-8.3% relative to control cells, but the difference between them was not statistically significant. Pretreatment with 20μM curcumin, prior to FP-12 also had no significant effect on cell viability, the viability of the cells remained at 93.3 +/- 22.5%, when measured by the students t test, (significance reached when P<0.05).
Figure 5.1: Effect of curcumin and resveratrol on HCEC cell viability as determined by the MTT assay. HCEC cells were exposed to 10ng/ml TNFα or 20μM FP-12 for 24 hours with or without pretreatment for 1 hour with 10-40μM curcumin (A) or resveratrol (B). The cell viability is expressed as a percentage of untreated cells (viability = 100%). The data represents the mean +/- SD of three determinations. Statistical significance was determined (**p<0.005; ***p<0.001) by analysis of variance (student’s t test) where cells exposed to FP-12 or TNFα after curcumin pretreatment, differ significantly from cells exposed to TNFα or FP-12 alone.
However pretreatment with 40µM curcumin prior to TNFα or FP-12 caused a significant reduction in cell viability, compared to untreated cells, down to 47.1 +/- 15.2% and 35.9% +/- 14.6% respectively.

In contrast to curcumin, pretreatment of HCEC cells with resveratrol (10, 20 or 40 µM) for 1 hour prior to 10ng/ml TNFα or 20µM FP-12 for 24 hours had no significant affect on cell viability at any of the concentrations tested, (Figure 5.1B).

5.3: Effect of FP-12 on the morphology of HCEC cells

During apoptosis the integrity of the plasma membrane remains intact. Apoptotic cells exclude non-vital dyes such as trypan blue or propidium iodide (PI), while necrotic cells do not (Vermes et al, 1995). Therefore to discriminate between apoptotic and necrotic cells, HCEC cells were stained with the vital DNA stain Hoechst (HO) 33342 and PI simultaneously (section 2.20). The PI induces an intense red fluorescence of the DNA in cells with damaged cell membrane (necrotic cells) while it is excluded by apoptotic cells. Staining with HO33342 results in blue fluorescence of the DNA present in vital cells.

It was noticed that after exposure of HCEC cells to FP-12 alone, for 24 hours, some cells became detached from the tissue culture dish. Since these cells could be apoptotic, the effect of FP-12 on the levels of apoptosis was determined. Confluent HCEC cells, grown on coverslips in 6 well multi-dishes, were exposed to 20µM FP-12 for various times, between 4 and 24 hours prior to being stained with HO33342 and PI. HCEC cells were also exposed to H₂O₂ (0.5 and 1mM) for similar incubation times to FP-12. The treatment with H₂O₂ was to act as a positive control, since it has previously been shown that exposure to H₂O₂ induces apoptosis in a variety of cell types, thereby directly establishing oxidative stress as a mediator of apoptosis (Slater et al, 1995).

The cells were viewed under a fluorescence microscope, and the number of apoptotic and non-apoptotic cells in a given field was counted based on morphology. Cells with condensed chromatin were considered apoptotic. For each treatment 10 random fields of cells were counted, that contained at least 500 cells in each field. The percentage apoptotic cells was determined before calculating the mean percentage apoptotic cells
for each treatment. The student’s t-test was used to determine the significant differences between means, of percentage apoptotic cells in the exposed population compared with the control population. P values less than 0.05 were taken as statistically significant (*= P<0.05; **= P<0.01; ***= P<0.001).

As time of exposure to FP-12 (20µM) increased from 4 to 12 hours, the number of cells observed as being apoptotic also increased (Table 5.2). After exposure to FP-12 for 4 hours there appeared to be condensed pre-apoptotic cells. Up to 12 hours of exposure to FP-12 the percentage of apoptotic cells observed increased and was significantly greater than that observed in untreated cells. Exposure to FP-12 for longer time points (16 and 24 hours) did not cause a significant increase in the percentage of apoptotic cells in the treated cells compared to the untreated cells.

H₂O₂ induced the cells to undergo apoptosis more potently than FP-12; after 4 hours of exposure to FP-12 the mean percentage of apoptotic cells was 1.1% compared to 1.8% in H₂O₂ treated cells, and after 12 hours the percentage apoptotic cells was 1.9% in FP-12 treated cells, and 49.4% in H₂O₂ treated cells. When cells were exposed to H₂O₂ for longer time points (16 and 24 hours) the H₂O₂ concentration was reduced from 1mM to 0.5mM, since it appeared at the higher concentration, even after exposure for 12 hours, most of the cells became detached from the cover-slip, so the total number of cells counted was lower than in the other treatments. When the cells were stained with PI as well as Hoechst, to determine if any of the cells appeared necrotic, none of the treated cells at any of the time points appeared to be stained with PI, suggesting that exposure to 20µM FP-12, alone does not cause the cells to become necrotic.

H₂O₂ is thought to possibly activate apoptosis by inducing oxidative stress in cells. It is possible that FP-12 may induce apoptosis by producing reactive oxygen intermediates, and causing oxidative stress within the cells. Reactive oxygen mediated DNA damage has been shown to elicit the activation of poly-ADP-ribose transferase (Boorstein et al, 1995). This enzyme causes the polymerisation of ADP-ribose to proteins, which causes a rapid depletion of cellular NAD/NADH pools and a depletion of ATP stores, and as a consequence cell death.
## Table 5.2: Effect of FP-12 or H$_2$O$_2$ on percentage apoptotic HCEC cells counted in randomly selected populations of cells.

HCEC cells were grown on coverslips to confluency in 6 well multi dishes, and then exposed to FP-12 (20μM) or H$_2$O$_2$ (0.5 or 1mM) for 4-24 hours. After treatment the cells were stained with PI and Hoechst 33342, viewed under a fluorescent microscope and the number of normal and apoptotic cells in 10 randomly chosen populations were counted. The student’s t test was used to determine if the percentage apoptotic cells was significantly increased in cells exposed to FP-12 or H$_2$O$_2$ compared to unexposed, control, cells (*p<0.05, ** p<0.01, *** p<0.005).

To investigate the possible role of poly-ADP ribosylation in the increase in apoptosis by FP-12, the percentage of apoptotic cells in HCEC cells exposed to FP-12 in the presence and absence of 3 amino-benzamide (3AB), an inhibitor of poly-ADP-ribosylation, was

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<thead>
<tr>
<th>TREATMENT</th>
<th>EXPOSURE TIME (hours)</th>
<th>MEAN PERCENTAGE APOPTOTIC CELLS</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>4</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>FP-12 (20μM)</td>
<td>4</td>
<td>1.1</td>
<td>p&lt;0.01 (**)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.0</td>
<td>p&lt;0.001 (***)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.9</td>
<td>p&lt;0.0005 (****)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.28</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.32</td>
<td>p&gt;0.1</td>
</tr>
<tr>
<td>H$_2$O$_2$ (1mM)</td>
<td>4</td>
<td>1.8</td>
<td>p&lt;0.0005 (****)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>not measured</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>49.4</td>
<td>p&lt;0.0005 (****)</td>
</tr>
<tr>
<td>H$_2$O$_2$ (0.5mM)</td>
<td>16</td>
<td>0.43</td>
<td>P&gt;0.025 (*)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>no cells detached</td>
<td></td>
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</tbody>
</table>
investigated. Cells were treated with 20µM FP-12, in the presence or absence of 25mM 3AB. In control, untreated cells 3AB did not alter the background percentage apoptotic cells counted. Exposure of HCEC cells to 20µM FP-12, for 4 hours, gave a mean percentage apoptosis of 1.1% which was not significantly reduced, as determined from the student’s t test, in HCEC cells also exposed to 3AB (1.1% compared to 0.7%). After exposure to FP-12 in the presence of 3AB for 16 hours, the mean percentage apoptotic cells was 0.30%, compared to 0.28% in cells exposed to FP-12 alone. This suggests that FP-12 did not induce apoptosis due to the induction of oxidative stress within the cells since 3AB did not have any significant effect on the percentage number of apoptotic cells measured.

5.4: Effect of pretreatment of HCEC cells with curcumin, prior to TNFα or FP-12 on PS externalisation

To determine to what extent the loss of cell viability in HCEC cells pretreated with curcumin (40µM) prior to FP-12 or TNFα was due to apoptosis, a method involving annexin was used, to distinguish between possible loss of cell viability due to apoptosis or necrosis (section 2.18). Because externalisation of PS from the inner to the outer leaflet of the plasma membrane occurs in the earlier stages of apoptosis, Annexin V-FITC staining, which binds to cells with surface exposed PS, can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. If PI is also added to the cells it is possible to distinguish between cells which are in the early stage of apoptosis from those that are in the later stages of apoptosis or already dead. Viable, non-apoptotic cells will contain neither stain. Apoptotic cells, with intact membrane integrity are stained only by Annexin V-FITC. Cells in the later stages of apoptosis or in a secondary necrosis phase, consecutive to apoptosis, in vitro, contain both stains, PI and Annexin V-FITC (Martin et al, 1995, Vermes et al, 1995), since their plasma membrane integrity is lost and is therefore permeable to PI.

HCEC cells were exposed to 40µM curcumin for 1 hour, prior to 10ng/ml TNFα or 20µM FP-12 for either 7, 16 or 24 hours. After treatment the cells were incubated in a buffer containing Annexin V-FITC and PI, prior to being run on a flow cytometer with a 488nm laser as the light source. The cells, observed as a scattergram on the flow cytometer, were divided into 4 quadrants; the lower left quadrant contain the viable cells
Figure 5.2: Probing for phosphatidyl-serine redistribution. Dot blots of FITC-Annexin V/PI by flow cytometry of HCEC cells after exposure to 10ng/ml TNFα or 20μM FP-12, for 16 hours, with or without 1 hour pretreatment with 40μM curcumin. After treatment the cells were incubated in a buffer containing Annexin V/FITC and PI, and analysed by flow cytometry. The results shown are representative of two experiments.
negative for both the FITC-Annexin V binding and PI uptake. The lower right quadrant represents apoptotic cells positive for Annexin binding and negative for PI uptake, demonstrating cytoplasmic membrane integrity. The upper right quadrants contain secondary apoptotic or necrotic cells, positive for both FITC-Annexin V binding and for PI uptake.

At each time point investigated 7, 16 and 24 hours, in control cells in the absence of any induced apoptosis, there was a small proportion of cells that were positive for Annexin uptake (0.6-1.1%) and cells positive for both Annexin V binding and PI uptake (9.8-13.2%) (Table 5.3). This is taken as the basal level of apoptotic and dead cells in the untreated cell population. However most of the untreated cells were located in the lower left quadrant, Annexin V and PI negative, indicating that the cells were viable, and not undergoing apoptosis (81.4-82.8% of cells).

Exposure to 10ng/ml TNFα and 20μM FP-12 alone for 7, 16 or 24 hours had little effect on the frequency of apoptotic or dead cells, ie, only background levels of necrosis/ secondary apoptosis were detected, and no increase in apoptosis or necrosis was observed over time. Pretreatment of HCEC cells with 40μM curcumin prior to FP-12 or TNFα for 7 hours showed a similar pattern to control cells, with no increase in apoptosis. However after pretreatment with 40μM curcumin prior to FP-12 or TNFα for 16 hours, the majority of the cells were either undergoing apoptosis (in the bottom right quadrant) or were already dead (top right quadrant) (Table 5.3). This is further illustrated in scattergrams of FITC-Annexin V/ PI stained cells, (Figure 5.2). After 24 hours exposure to FP-12 or TNFα, in the presence of curcumin, fewer cells stained positive for Annexin only, indicating that cells were not undergoing early apoptosis (above that seen in control cells). A high proportion of cells (~35-40%) stained positive with Annexin and PI; about the same percentage as seen after exposure for 16 hours to the test agents.
Table 5.3: Effect of curcumin pretreatment on the induction of apoptosis and necrosis, measured by PS externalisation and PI staining, in HCEC cells subsequently exposed to TNFα or FP-12. Cells were exposed to 10ng/ml TNFα or 20μM FP-12 for 7, 16 or 24 hours, with or without 1 hour pretreatment with 40μM curcumin. After treatment the cells were incubated in a buffer containing Annexin V-FITC and PI. Normal cells contained neither stain, apoptotic cells stained with Annexin V-FITC and secondary apoptotic (necrotic) cells stained with both Annexin V-FITC and PI.

5.5: Effect of curcumin on cell cycle distribution

The proportion of cells in each phase of the cell cycle was determined by using flow cytometric analysis (section 2.19). Apoptotic cells are distributed in the sub-G0/G1 peak, showing a diminished staining with the DNA specific fluorochrome, PI, below the G0/G1 population. The reduced DNA content is a consequence of apoptosis. In apoptotic cells there is a progressive loss of DNA from the nuclei due to the activation of an endogenous endonuclease, and subsequent leakage of the low molecular weight DNA product prior to measurement. A sub-diploid peak in DNA content correlates with morphological and biochemical hallmarks of apoptosis. In contrast to apoptotic cells, necrotic cells do not show an immediate reduction in DNA content. When a cell is triggered to enter the cell cycle it moves out of G0 and into the G1 phase of the cell cycle. During this phase DNA levels increase and certain proteins essential for replication of DNA are made. The cell starts to replicate its DNA during S phase (a DNA synthetic phase of the cell cycle), and when the DNA content has doubled DNA
synthesis ceases and the cell enters into G2 phase. The cell divides through M phase returning to the start of G1.

Flow cytometric analysis of HCEC cells after exposure to 10ng/ml TNFα or 20μM FP-12, for 24 hours, with or without pretreatment with 40μM curcumin for 1 hour, showed a cell cycle distribution, after staining the DNA with PI, as illustrated in Figure 5.3. When DNA content was plotted against the number of cells, as shown, the percentage number of cells in each phase of the cell cycle was determined. With all of the treatments, with or without pre-treatment with curcumin, the percentage number of cells with their DNA content in the sub-diploid fraction (sub-G0/G1), did not change compared to untreated cells (Table 5.4). In all of the treatments compared to control cells; there was an increase in the population of cells in G2/M and a decrease in the population of cells in G0/G1. However curcumin pretreatment prior to TNFα or FP-12 did not alter the distribution of cells in the cell cycle. S phase cells were not clearly seen in this experiment, but were probably distributed between M2 and M3 (G0/G1 and G2/M), since cells in S phase have a DNA content lying between these extremes.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>SUB-G0/G1 (M1) % total cell no.</th>
<th>G0/G1 (M2)</th>
<th>G2/M (M3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.28</td>
<td>46.64</td>
<td>43.37</td>
</tr>
<tr>
<td>TNFα (10ng/ml)</td>
<td>8.47</td>
<td>30.58</td>
<td>61.41</td>
</tr>
<tr>
<td>TNF + curcumin</td>
<td>10.80</td>
<td>30.95</td>
<td>58.78</td>
</tr>
<tr>
<td>FP-12 (20μM)</td>
<td>9.27</td>
<td>28.71</td>
<td>63.79</td>
</tr>
<tr>
<td>FP-12 +curcumin</td>
<td>9.81</td>
<td>29.61</td>
<td>60.91</td>
</tr>
<tr>
<td>Curcumin (40μM)</td>
<td>9.43</td>
<td>35.69</td>
<td>56.47</td>
</tr>
</tbody>
</table>

Table 5.4: Effect of curcumin pretreatment on cell cycle phase distribution of HCEC cells exposed to FP-12 and TNFα. HCEC cells were exposed to 10ng/ml TNFα or 20μM FP-12 for 24 hours, with or without 1 hour pretreatment with 40μM curcumin. The proportion of cells in each phase of the cell cycle was determined with flow cytometry analysis of PI stained cells. The percentage apoptotic cells is shown in the sub-G0/G1 region. Representative of 2 experiments.
Figure 5.3: Determination of cell cycle distribution and apoptosis in HCEC cells by DNA content analysis of PI stained cells. Cell cycle phase distribution was determined by flow cytometry, where PI was used to label the DNA. The figure shows flow cytometry profiles, showing DNA content (cell number) histograms, of cells exposed to 10ng/ml TNFα or 20μM FP-12 for 24 hours with and without pretreatment for 1 hour with 40μM curcumin. M1=sub-G0/G1; M2=G0/G1; M3=G2/M. The results are representative of two experiments.
5.6: Effect of curcumin on the formation of DNA ladders

Since pretreatment with curcumin (40μM) was shown by Annexin/PI staining to increase cell death in HCEC cells exposed to 10ng/ml TNFα the effect of these agents on internuclearosomal DNA fragmentation was examined in HCEC cells. Total DNA was extracted and subjected to agarose gel electrophoresis to look for DNA laddering. Treatment with curcumin alone, or as a pretreatment prior to TNFα or FP-12 exposure for 24 hours, did not produce the characteristic ladder of oligonucleosomal DNA fragments of 200 base pair integer multiples in size. This suggests that there was no evidence of any preferential DNA degradation at the inter-nucleosomal linker DNA regions in the cells (Figure 5.4). However, although there was no cleavage of DNA at regular intervals, as is the fragmentation pattern for apoptosis, no random fragmentation associated with necrosis was observed.

5.7: Discussion

Apoptosis results from activation of a pre-programmed pathway of biochemical events leading to cell death (Wright et al, 1994). A large body of evidence indicates that apoptosis may represent a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells that have improperly been induced to divide, by factors such as carcinogens (Payne et al, 1995). Inhibition of apoptosis is one mechanism of tumour formation, and many chemopreventive agents may act through the induction of apoptosis in the target tumour cells to block the carcinogenic process. Apoptosis plays an important role in the reduction of cell numbers by chemopreventive agents such as NSAIDs in colorectal cancer cell lines (Shiff et al, 1996). Curcumin could exhibit pronounced anti-tumour activity by triggering cell death in human tumour cells. Kuo et al (1996) found that curcumin exhibited a remarkable cytotoxic effect on various cancer cells including HeLa (cervical carcinoma), SW620 (colon cancer), MCF-7 (Breast cancer) and HL60 (leukemia) cell lines. Curcumin increased the percentage of apoptotic cells and reduced tumour incidence by 50% in colon tumours of rats exposed to AOM. Samaha et al (1997) showed in an in vivo model system that dietary administration of curcumin induces apoptosis in colonic tumours of male F344 rats. Suppression of cell death by tumour promoting agents, such as TNFα and FP-12, in preneoplastic cells could be an important mechanism of tumour
Figure 5.4: Agarose gel electrophoresis of HCEC cells exposed to TNFα or FP-12, with or without pretreatment with curcumin. HCEC cells were exposed to 10ng/ml TNFα or 20μM FP-12 for 24 hours with or without 1 hour pretreatment with 40μM curcumin. After treatment the cells were harvested, DNA extracted and analysed for DNA laddering by agarose gel electrophoresis. Lane 1, Hind III digest; lane 2, MW markers; lane 3, unexposed cells; lane 4, 10ng/ml TNFα exposed cells; lane 5, TNFα + 40μM curcumin; lane 6, 40μM curcumin exposed cells; lane 7, 20μM FP-12 exposed cells; lane 8, FP-12 + 40μM curcumin exposed cells.
promotion, therefore it can be suggested that the anti-tumour promoting effects of curcumin may at least in part be contributed to its ability to induce apoptosis. In addition, Tsujji and Dubois (1995) who have implicated COX-2 activity in the regulation of apoptosis in rat intestinal epithelial cells, have demonstrated that overexpression of COX-2 can cause the cells to adhere more to the extracellular matrix, and make them resistant to apoptosis. Gallois et al (1998) demonstrated that in human hepatic cells, activation of COX-2 is a key event in the pathway leading to human stellate cell proliferation. Since curcumin has been shown to inhibit both COX-2 gene expression and NF-κB activation in HCEC cells, the effect of curcumin on apoptosis was investigated to determine the biological consequences of these effects on the cells.

Several observations indicate that oxidative stress is an important mediator of apoptosis for example ionising and UV radiation, low concentrations of H₂O₂ and certain cytokines stimulate apoptosis and generate reactive oxygen species (Hockenbery et al, 1993). The results of the Hoechst staining for apoptotic morphology shows that as time of exposure to FP-12 increased from 4-12 hours, there was an increase in apoptotic cell death which reached significance compared to unexposed cells. However after 24 hours exposure to FP-12 the percentage of apoptotic cells was not significantly different from control cells, which could be due to compensatory growth stimulation after prolonged incubation with FP-12. FP-12 may induce apoptosis of normal undamaged cells thereby selecting cells that have damaged DNA with increased growth characteristics.

Since curcumin has been shown to have a marked effect on COX-2 mRNA expression it is important to understand the possible biological effects of this phenomenon. This could include the regulation of the dynamics of cell proliferation and cell death in colon mucosa. It appeared that when cell viability was measured after 24 hours, by trypan blue exclusion method, there appeared to be a correlation between the degree of inhibition of NF-κB activation and the extent of cell death. Exposure of HCEC cells to TNFα (10ng/ml) or FP-12 (20μM) alone, caused no reduction in cell viability, treatments that induced NF-κB DNA binding activity. Exposure to curcumin alone also had no effect on cell viability. Pretreatment for 1 hour with 10μM curcumin showed no marked reduction in cell viability or NF-κB DNA binding, whereas 40μM curcumin, a concentration that markedly inhibited NF-κB binding induced by TNFα, caused a reduction in cell viability down to 4.4 (+/- 1.7)% when measured by trypan blue.
Pretreatment with curcumin (40μM) prior to FP-12 or TNFα for 24 hours also caused a significant decrease in cell viability compared to cells exposed to FP-12 or TNFα alone, as measured by the MTT assay. TNFα alone did not produce significant cytotoxic effects in HCEC cells but pretreatment with curcumin (40μM) caused the cells to be highly responsive to TNFα, producing greatly increased levels of cell death. Therefore stimuli that cause activation of NF-κB may inhibit cell death whereas inhibition of NF-κB serves as a trigger for cell death to occur. This observation is consistent with the data of Liu et al (1996) showing that NF-κB activation can inhibit apoptosis induced by TNFα. In contrast, as observed in chapter 4, resveratrol had no inhibitory action on NF-κB DNA binding activity. Pretreatment for 1 hour with 10, 20 or 40μM resveratrol, prior to TNFα (10ng/ml) or FP-12 (20μM) for 24 hours, caused no significant reduction in cell viability. This could explain why resveratrol had no inhibitory activity on NF-κB DNA binding if NF-κB activation causes resistance to apoptosis. The results of the present study show that in cells exposed to TNFα in the presence of resveratrol, there was minimal toxicity to the cells after 24 hours exposure. The observations in the present study are similar to those of Clement et al (1998) who observed that resveratrol had minimal toxicity to normal human peripheral blood lymphocytes, and had no toxicity in HL60 cells, a human promyelocytic leukemia cell line and human breast carcinoma cell line T47D. However Huang et al (1999) found that the induction of apoptosis was involved in the anticarcinogenic effects of resveratrol in JB6 cells.

To determine whether the effects of curcumin on cell viability were mediated by apoptosis or necrosis, an Annexin method, sub-G1 determination and a DNA laddering method were used. The problem with the results of the Annexin V assay is that the assay does not distinguish between cells that have already undergone an apoptotic cell death and those that have died as a result of a necrotic pathway, because in either case the dead cell will stain with both Annexin and PI. One limitation of using this assay to determine if curcumin has an effect on cell death in HCEC cells, is that as these are adherant cells and grow as a monolayer, the cells need to be trypsinised after treatment for analysis. Trypsin may interfere with the Annexin binding results. In addition PI uptake could be the expression of a non-specific effect of curcumin on HCEC cell membrane structure, as observed by Jaruga et al (1998). These authors suggested that curcumin partitioning into the lipid bilayer could result in alterations of lipid packing.
and/or transbilayer motion of the lipids, thus increasing the basal permeability of the membrane. This observation would explain why the HCEC cells stained with both PI and Annexin.

Khafif et al (1998) showed by flow cytometry that curcumin blocked cells in S/G2M in normal oral epithelial cells. Hanif et al (1997) also observed that curcumin altered the cell cycle distribution of HT-29 and HCT-15 cells, two human colon adenocarcinoma cell lines, causing a time and concentration dependent accumulation of cells in the G2/M phase of the cell cycle, but no sub-G1 fraction. However in the experiments of this study with HCEC cells using a flow cytometry technique, with PI staining, curcumin had no effect on cell cycle distribution compared to untreated cells. Sikora et al (1997) noted that when curcumin was given to rat thymocytes at 50μM concentration for 24 hours no sub-G1 fraction was observed. Differences in results could be due to the different model systems used or different experimental protocols.

From the results of the present study it appears that curcumin does increase cell death in cells exposed to the tumour promoters FP-12 and TNFα, but this is probably not cell death by apoptosis. However the induction of apoptosis by curcumin cannot be ruled out completely. The degradation of genomic DNA, another marker of apoptosis, was also analysed to measure the apoptotic response of HCEC cells to curcumin treatment. Although curcumin did not induce DNA ladders in HCEC cells, there is the possibility that curcumin could induce apoptosis in these cells. Curcumin may interact with the endonucleolytic activity by preventing internucleosomal DNA cleavage, so DNA ladders would not be formed, but may not protect against the formation of high molecular weight fragments (Piwocka et al, 1999). Kuo et al (1996) observed typical DNA ladders after curcumin treatment of HL60 cells. Although non-random DNA fragmentation is widely used as a marker of apoptosis, it is important to verify the occurrence of apoptosis by other criteria such as cell morphology (Wolfe et al, 1996), since not all cells exhibit the characteristic DNA fragmentation of internucleosomal fragments (Oberhammer et al, 1993).

Curcumin has been shown, by a non-quantitative method, to induce apoptosis in HT-29 colon cancer cells (Jiang et al, 1996b). However Hanif et al (1997) reported that in HT-29 and HCT-15 colon adenocarcinoma cell lines that curcumin failed to induce
apoptosis, indicating that curcumin reduced the cell number by an exclusively or predominantly antiproliferative effect. Chen and Huang (1998) exposed A7r5 cells, a rat aortic smooth muscle cell line to curcumin, and saw no effect on the levels of apoptosis when the cells were exposed to curcumin at concentrations less than $10^{-4}$M. Different results from the present study on the effect of various treatments on cell death compared to what others have reported with similar treatments may reflect different susceptibilities of the various cell lines for curcumin induced apoptosis. If curcumin can induce apoptosis in HCEC cells, it may be of a limited extent and has to be detected by more sensitive methods, such as the comet assay or the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) technique.
CHAPTER 6: GENERAL DISCUSSION
6.0: Discussion

To develop an effective approach for suppressing the expression of the COX-2 gene, it is important to determine the signalling mechanisms that govern its increased expression. Among the possible target genes of the transcription factor NF-κB is COX-2 since this gene has two consensus binding sites for NF-κB in its promoter region (Yamamoto et al, 1995), which are absent from that of the COX-1 gene. The results of the effect of exposure of HCEC cells to FP-12 and TNFα on COX-2 gene expression (Chapter 3, section 3.3) and NF-κB activation (Chapter 4, section 4.2) suggests that upregulation of COX-2 gene expression by these two tumour promoters requires NF-κB activation in HCEC cells, a normal colon epithelial cell line. This study demonstrates that the colon tumour promoters TNFα and FP-12 cause activation of both an NF-κB consensus sequence and activation of a specific NF-κB binding sequence found in the COX-2 promoter region (Chapter 4, section 4.4) and with the same concentrations of agents and time frame, induction of COX-2 gene expression. The results of the present study are in accordance with other recent observations implicating NF-κB in COX-2 activation by interleukin-1, TNFα or lipopolysaccharide, by means of p65 antisense or NF-κB inhibitors (Yamamoto et al, 1995; Crofford et al, 1997; Hwang et al, 1997). Jobin et al (1998) have recently shown that NF-κB is critical for the induction of COX-2 gene expression by TNFα in HT-29 cells, a human colon tumour cell line. However regulation of COX-2 gene expression by multiple signalling pathways would be expected on the basis of the complexity of its promoter region. Other transcription factors have been shown to be important for COX-2 induction. A functional requirement for the CRE site of the human COX-2 promoter has been shown to be necessary for COX-2 induction of expression in differentiation of U937 cells by phorbol esters (Inoue et al, 1994). The importance of both the CRE and NF-IL6 sites for COX-2 expression in LPS and phorbol ester stimulated human vascular smooth muscle cells has been described (Inoue et al, 1995). Since TNFα also activates AP-1 (Brenner et al, 1989) it is possible that this could contribute to the effects of TNFα on COX-2 expression.

It is possible that FP-12 and TNFα activate NF-κB through distinct signalling pathways. Exposure to TNFα leads to the degradation of IkB (Chapter 4, section 4.8), suggesting
an involvement of an IKK kinase, whereas FP-12 activated NF-κB without degrading IkB. It is unlikely that FP-12 activates the NIK/IKKα pathway at the level of the TNF receptor. It has been shown previously that FP-12 induces oxidative stress in cells (Plummer and Faux, 1994). As ROS are a common denominator in NF-κB activating signals, this may underly the effects of FP-12 on the NIK/IKKα pathway. FP-12 was shown to cause a slight activation of PKC in HCEC cells (Chapter 4, section 4.9) which is also a known pathway to activate NF-κB, whereas TNFα has been reported not to have an effect on PKC activity (Meichle et al, 1990). This suggests that NF-κB can be activated by multiple signalling pathways in HCEC cells, by different tumour promoters.

A typical Western diet may be associated with increased incidences for colon cancer due to the high levels of endogenous tumour promoters such as fecapentaenes and bile acids in the colon. Individuals found to excrete high levels of fecapentaenes appear to be at a decreased risk of developing colorectal neoplasia (de Kok et al, 1992). Although fecapentaenes have been shown to possess tumour promoting activity in animal models of colon cancer (Zarkovic et al, 1993), this is the first in vitro report of the effect of FP-12 on the induction of early response gene expression. From the results of the present study it is proposed that FP-12 could have a promoting role in the development of colon cancer, possibly by increasing COX-2 expression via activation of NF-κB. This is of importance since the results observed from animal studies were not conclusive. The use of rodents to study the effects of fecapentaenes may not be a sensitive enough model system to use, since Weisburger et al (1990) and Ward et al (1988) observed negative carcinogenicity data for fecapentanes in the rat colon. Fecapentanes may not be acting as carcinogens in the rodent model due to insufficient uptake. The only animal besides man which has been shown to produce fecapentaenes endogenously is the mini-pig (Pertel, 1986). Therefore rodent studies may not be a suitable in vivo model to use to study the carcinogenic mechanisms of fecapentaenes, suggesting an in vitro system using human colon cells could be more informative. A more detailed understanding of the precise mechanisms by which fecapentaenes induce COX-2 expression should facilitate the development of chemopreventive strategies to diminish the risk of carcinogenesis within regions of the gastrointestinal tract exposed to fecapentaenes.
That curcumin can act as a chemopreventive agent has been demonstrated in various animal tumour models. For example curcumin has been shown to cause a 50% decrease in the tumour incidence in rats exposed to AOM (Samaha et al, 1997). The results presented here demonstrate that the dietary constituent curcumin inhibits the activation of NF-κB (Chapter 4, section 4.2) and expression of the COX-2 gene (Chapter 3, section 3.4.2) induced by TNFα and FP-12 in HCEC cells. Several of the chemopreventive properties of curcumin can be explained by its ability to inhibit COX-2, for example curcumin suppresses the formation of adducts between metabolites of benzo [a] pyrene and DNA (Huang et al, 1992) possibly by inhibiting COX, since COX converts a range of xenobiotics to mutagens. The ability of curcumin to inhibit tumour promoter induced COX-2 gene expression may be due to its inhibitory action on NF-κB, since this transcription factor has been shown to be important in the induction of COX-2 gene expression, as noted above. Since curcumin also inhibited NF-κB activation by H₂O₂ (chapter 4, section 4.6) this suggests that curcumin is a general suppressor of NF-κB activation. However it has not yet been identified what members of the signalling cascade leading to the activation of COX-2 are the targets for curcumin. Since curcumin is a kinase inhibitor curcumin may inhibit NF-κB activation by inhibiting a kinase that controls IkB stability. For example it has been reported that the release of NF-κB from IkB is initiated by serine phosphorylation through two kinases, IKKa and IKKB (DiDonato et al, 1997). Recent studies in our laboratory have provided indirect evidence that curcumin inhibits NF-κB activation at the level of IKK (Plummer et al, 1999, in press). Activation of IKK can be initiated by several pathways including the cellular kinases, NIK and MEKK1 (Lee et al, 1997). Guan et al (1998) reported a signalling pathway to activate COX-2 involving MEKK1, MKK4 and p38 MAPK. Curcumin has been reported to inhibit PKC activity (Liu et al, 1993) and in a separate study, AP-1 DNA binding (Zhang et al, 1999). Therefore part of the inhibitory effects of curcumin on COX-2 gene induction by TNFα and FP-12 may be mediated by this target.

The results of the present study demonstrate a possible link between inhibition of HCEC cell growth by inducing cell death and inhibition of the COX-2 signalling pathway, possibly at the level of NF-κB activation. Overexpression of COX-2 occurs at an early stage in colon carcinogenesis, and can cause resistance to apoptosis in colon epithelial cells (Tsujii and Dubois, 1995). It is conceivable that cells continuously sustain their
growth after stimulation with FP-12 or TNFα by using the extracellular PGE2 or other
prostaglandins that they themselves produce via COX-2 induction, and in turn this
upregulates the expression of COX-2 and other growth related genes (Tjandrawinata et
al, 1997). This suggests that treatment with curcumin might reinstate the susceptibility
to apoptosis. Samada et al (1997) reported that treatment with curcumin stimulates
apoptosis in the colon. The results presented here, in HCEC cells are in agreement,
suggesting that one mechanism for the chemopreventive effect of curcumin against
colon cancer is mediated at least in part by its ability to decrease COX-2 mRNA
expression, via inhibition of the NF-κB signalling pathway. This in turn increases the
cells susceptibility to cell death, possibly by apoptosis and decreases the rate of cell
proliferation. This is consistent with studies which show that NF-κB activation is
essential for cell survival (Baichwal and Baeuerle, 1997), ie NF-κB controls the
transcriptional activity of a gene(s) which induces the synthesis of survival proteins.
However the role of NF-κB in the induction of cell death is still not yielding a
homogenous picture, with reports of both pro- and anti-apoptotic aspects in the
literature. Overexpression of the NF-κB family member c-Rel arrests the proliferation
of Hela cells by interacting with p21^{WAF1} (Bash et al, 1997). In contrast, the inhibition of
NF-κB activation promotes TNF-α mediated cell death in HeLa cells, macrophages,
fibroblasts, fibrosarcoma cells and Jurkat cells (Beg and Baltimore, 1996), while it is
ineffective in other cell lines such as human breast carcinoma cells (Cai et al, 1997).
Part of the discrepancies may be explained by differences in the cell types studied and
the nature of the apoptosis inducing stimulus. Although the mechanism of the cell
protecting effect of NF-κB is still not clear, it might rely on the induced expression of
anti-apoptotic genes, such as COX-2, or other genes that could be likely candidates, that
were not studied such as the zinc finger protein A20 (Opipari et al, 1992; Cooper et al,
1996) or manganese superoxide dismutase (Wang and Goeddel, 1988; Baichwal and
Baeuerle, 1997). It has recently been demonstrated that NF-κB is needed for TNFα
mediated induction of IAP-2, a protein of the inhibitor of apoptosis (IAP) protein
family. When overexpressed in mammalian cells IAP2 activates NF-κB and suppresses
TNFα cytotoxicity (Chu et al, 1997). Since COX-2 is an NF-κB activated anti-apoptotic
gene, curcumin which prevents the translocation of NF-κB to the nucleus, by inhibiting
the degradation of IκBα, may induce cell death by blocking COX-2 expression.
Although chemopreventive agents comprise a diverse group of compounds with
different mechanisms of action, their ultimate ability to increase the rate of cell death and decrease cell proliferation, may represent a unifying concept for the mechanism of chemoprevention. Understanding the modes of action of these compounds should provide useful information for their possible application in cancer prevention and perhaps also in cancer therapy.

In the present study the effects of TNFα on an NF-κB signalling pathway has been examined, but it has to be remembered that TNFα receptor binding stimulates at least two distinct signal transduction pathways (Hsu et al, 1996). TNFα itself can stimulate apoptosis via interaction of the TNFα receptor complex and the Fas associated protein with a death domain. Secondly TNFα can activate JNK and the transcription factor AP-1, a transcription factor that is important in the induction of COX-2 gene transcription by v-src (Xie and Hershman, 1995). Since curcumin can inhibit the activation of AP-1 (Huang et al, 1991b), some of the inhibitory effects of this agent on COX-2 gene induction by TNFα could be mediated not only by the inhibition of NF-κB but also of this transcription factor.

Feng et al (1995) demonstrated that hydrogen peroxide and superoxide are capable of inducing COX-2 mRNA and that this induction can be inhibited by free radical scavengers. Hempel et al (1994) reported that antioxidants might interfere with the ability of DNA binding proteins, such as NF-κB to interact with the human COX-2 promoter. It is unlikely that the inhibitory effects of curcumin observed on NF-κB translocation and activation, COX-2 mRNA expression and on the increase in cell death is due to its non-specific antioxidant activity. NAC, a classical antioxidant, increased COX-2 mRNA expression by TNFα and FP-12 and NAC alone, caused a 2 fold increase in COX-2 mRNA expression (Chapter 3, section 3.4.5). This could be because NAC may not always inhibit NF-κB activation in all cell types, as seen by Brennan and O’Neill (1995) who reported that NAC did not inhibit NF-κB activated by IL-1α. Since antioxidants have been shown to stimulate AP-1 activation (Meyer et al, 1993) it could be that the increase in COX-2 gene expression by NAC may be due to its ability to induce AP-1 activation via the CRE element in the promoter region of the COX-2 gene. The inducible effects on gene expression and factor binding to DNA regulatory sequences by phenolic antioxidants are highly complex, as indicated by a comparison of CAPE, with curcumin. CAPE, structurally related to curcumin, showed no inhibitory
effect on either the induction of NF-κB DNA binding (Chapter 4, section 4.2) or on COX-2 mRNA expression (Chapter 3, section 3.4.3) induced by TNFα and FP-12 at equimolar concentrations to curcumin. The fact that curcumin might not be acting solely as an antioxidant to prevent NF-κB activation by FP-12, suggests that intracellular ROS may be generated by this tumour promoter, but subsequent to its initial cellular effects.

The concentration of curcumin used to block COX-2 expression and NF-κB activation in these studies in vitro have been employed recently by other investigators (Mazumder et al, 1995; Chen et al, 1996; Kumar et al, 1998). Huang et al (1994) and Rao et al (1995b) have observed that curcumin has to be given at 0.2-5% (w/w) in the daily diet in animal studies, a concentration that is not likely to be adopted by most individuals in order to protect against colon cancer. However the concentration of curcumin in the range of 10-200mg/kg administered intraperitoneally or by gavage, has been used safely in animal studies to block carcinogenesis and inflammation (Huang et al, 1994; Rao et al, 1995). It is not conclusive what the bioavailability of curcumin is as an oral chemopreventive agent against cancer, mainly because in some of the carcinogenesis studies non-physiological levels of isolated compounds were administered to animals. Holder et al (1978) found that 90% of the oral dose of curcumin was excreted in the faeces within 48 hours. Available data on the metabolism and pharmacology of curcumin in humans is scarce, and there is no information on its potential as a chemopreventive agent in humans. Following oral consumption in humans, curcumin is probably absorbed poorly through the gastrointestinal tract, and is excreted in the faeces (Ammon and Wahl, 1991). Evidence suggests that low micromolar concentrations of curcumin can be achieved following oral consumption, of approximately 0.1mg/kg. The tissue levels of curcumin are unknown, but is likely that colon mucosal cells are exposed to high lumenal concentrations of curcumin. However it is uncertain whether the curcumin concentrations used in this study in vitro, can be achieved in vivo. The work of the present study does suggest possible mechanisms of chemoprevention that could be examined in more detail in appropriate animal or human model systems. Since the ability of curcumin to act as a chemopreventive agent in animal models of colon carcinogenesis has been shown (Pereira et al, 1996), it can be speculated that curcumin could be an important dietary agent for the chemoprevention of colon carcinogenesis in humans. Furthermore investigations of the mechanisms that determine the susceptibility
or resistance of various cell types to curcumin, may provide critical information for developing curcumin based novel anti-cancer or anti-inflammatory therapies.

There has been much need for the development of an alternative drug to aspirin as a chemopreventive agent in humans, since as many as 25% of individuals using NSAIDs experience some kind of side effect, and as many as 5% develop serious health consequences. Also in the present study, contrary to what has been noted by others, sodium salicylate showed no inhibitory effects on COX-2 expression, even though it has been shown to inhibit the degradation of IkB (Kopp and Ghosh, 1994), possibly by inhibiting the activity of IKKB (Yin et al, 1998) and inhibit COX-2 gene induction in various cell models, including HUVEC cells. An important element in the evaluation of the possible role of a chemopreventive compound is the assessment of preclinical toxicity. Unlike synthetic NSAIDs, curcumin does not produce any gastrointestinal toxicity, even at the very high doses, which may provide advantages over conventional NSAIDs. Although the literature on curcumin toxicity is scarce, curcumin has been used for centuries, in various populations with a lack of reported toxicity and side effects. The pharmacological safety of curcumin is demonstrated by its chronic consumption at levels of up to 100mg/day by people in certain countries without adverse effects (Ammon and Wahl, 1991; Kuo et al, 1996). Also anecdotal evidence suggests that patients who have received curcumin at 400mg, four times daily to treat inflammation, did not elicit toxicity (Ammon and Wahl, 1991). Consumption of dietary curcumin at approximately 1200mg/day has been reported to provide moderate relief to patients suffering from rheumatoid arthritis (Deodhar et al, 1980). From numerous studies in experimental animals it was clear that curcumin given orally at high doses did not cause apparent toxic effects. Previous studies on the toxicity of long term feeding of curcumin (8-60 weeks) to dogs, guinea pigs or monkeys, have indicated no evidence of histopathological changes or any teratogenic or carcinogenic effects. Also doses of curcumin (up to 5g/kg) have been found to have no adverse effects in rats (Wahlstrom and Blennow, 1978). Rats which received 750mg/kg/day of curcumin for 10 weeks did not reveal any toxic effects (Pulla Reddy and Lokesh, 1994). Curcumin is available in large quantities as a natural product. For these reasons further studies on curcumin are essential to test the colon cancer chemopreventive action of curcumin in clinical trials. In addition it needs to be determined whether the inhibitory effects of curcumin in vitro is mediated directly by the parent compound, or indirectly via one of its metabolites.
Nurfina et al (1997) reported that several symmetrically substituted curcumin derivatives were found to possess higher antiinflammatory activity than curcumin itself, particularly combinations of 4-hydroxy groups and 3,5-dialkyl groups. Sugiyama et al (1996) reported that the curcumin metabolite, tetrahydrocurcumin, is a stronger antioxidant in vitro than curcumin. However Huang et al (1995) found that pure curcumin and deoxycurcumin inhibit TPA induced tumour promotion whereas tetrahydrocurcumin was less effective. Isolation and testing of active substances not only provides naturally occurring novel agents as inhibitors of cancer development that may be more potent chemopreventive agents, but also offers opportunities to study the mechanisms of chemoprevention.

The importance of using chemopreventive agents which down regulate levels of COX-2 expression is that they will inhibit all functions of the enzyme and thereby may inhibit carcinogenesis more effectively than NSAIDs which only inhibit COX activity. Combining agents such as aspirin that inhibits the cyclooxygenase activity of COX with a compound, for example curcumin that suppresses the transcription of COX-2 may be more effective than using either agent alone. This idea is supported by a recent study in which the anti-tumour activity of a selective COX-2 inhibitor was potentiated by an antioxidant that inhibited the expression of COX-2 in vitro (Chinery et al, 1998). Since various classes of inhibitors interfere with NF-κB activation at different levels in the signal transduction cascade, it is tempting to speculate that combining some of the different agents could be highly effective as inhibitors of NF-κB. Furthermore the individual doses required to increase the chemopreventive effectiveness of the agents may presumably be reduced in such a mixture, thereby reducing or eliminating possible side effects and toxicity of the individual agents. Khaif et al (1998) showed a synergistic inhibition of growth was quantitatively demonstrated following treatment of normal, premalignant and malignant oral cells with a 1:1 combination of curcumin and epigallocatechin-3-gallate at different concentrations. Appropriate combinations of drugs could be established which would have limited toxicity against normal cells but have activity against premalignant or malignant cells.

There is now evidence from recent studies that COX-2 induction via NF-κB may not only be an important event in carcinogenesis. Recent studies have shown that COX-2 is induced by inflammatory cytokines, such as TNFα, which is produced in heart failure
via the transcription factor NF-κB (Yamamoto et al, 1995). In addition to cytokines, hypoxia, an important factor in ischemic myocardium, induces COX-2 (via NF-κB) in cultured endothelial cells, independently of other stimuli (Schmedtje et al, 1997). Wong et al (1998) showed induction of COX-2 and activation of NF-κB in the myocardium of the failing human heart. Therefore curcumin may not just be important as a chemopreventive agent, but may be important in preventing chronic heart failure.

The present studies have shown that curcumin inhibits the induction of COX-2 gene expression by two endogenous tumor promoters, TNFα and FP-12, and this is likely to contribute to its anti-inflammatory and chemopreventive activity. The high mortality with colorectal cancer is due in part to the fact that by the time symptoms have developed the cancer has become metastatic. The best hope to reduce the high mortality rates is to develop better screening and preventative measures to detect the disease earlier or prevent its initiation. Since COX-2 is over-expressed at an early stage in colon carcinogenesis, and is thought to play a causal role in the development of colon cancer, this suggests that curcumin could be a candidate for a chemopreventive agent against colon cancer. As has already been discussed it is reasonable to speculate that curcumin could possess several advantages over other synthetic NSAIDs, such as aspirin. Curcumin may function as a lead compound in the design of more effective and selective chemopreventive agents. Curcumin may not only act as a chemopreventive agent for colon cancer since Zimmermann et al, 1999, found constitutive COX-2 expressed in esophageal squamous cell carcinomas and suggests that COX-2 could be a new target in the chemoprevention of esophageal carcinoma. Selective COX-2 agents may be effective in other tumor types such as head and neck and breast cancer (Hwang, 1998). Further studies are needed to define the exact cellular signalling pathways through which curcumin inhibits COX-2 expression, particularly the kinases which are involved in the phosphorylation of IκB.


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