The Role of Protein Kinases in the Early and Late Stages of Colon Carcinogenesis

by

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Ted, Alexandra and Spike - you complete my world.

Abstract

The main objective of this research was to explore and examine the changes in mitogen activated protein kinases (MAPKs) in colonic tissue soon after carcinogen exposure and in colonic tumors. It is hypothesized that MAPKs play an important role in colon carcinogenesis and upregulation of the expression of MAPKs will provide an additional growth advantage to selected developing preneoplastic lesions. Two studies were conducted in male Sprague Dawley rats.

The first study explored the changes in risk markers such as cell proliferation, cell death and enzymatic parameters (MAPK, protein kinase C (PKC) and tyrosine kinase(TK)) in the colonic mucosa soon after carcinogen injection. Eighty Sprague-Dawley rats were fed the AIN-93 semi-synthetic diet and after 1 week 45 of the 80 animals were injected with azoxymethane (AOM). Nine injected and 7 control animals were terminated every 24 hours for the next five days.

The second study examined the expression of MAPKs in tumors as affected by a high calcium diet. In this study twenty of the twenty five animals were injected once a week for a three week period with AOM. All animals were fed a standard laboratory chow for twenty five weeks after which they were switched to a modified AIN-93 diet that contained either a normal level (0.5%) or a high level (2.0%) of calcium and fed this diet for a further three weeks.

The results of the first study demonstrated that the early events, as measured by cell proliferation, cell death and kinase activity were transient in nature and failed to elucidate any additional insights into their respective roles in colon carcinogenesis. The exception to this was the enzymatic activity of TK which was determined to be higher on day 5 in the membranous fraction. The activity of TK was also determined to be significant in the proximal colon. The results of the long term study revealed that there was a group of preneoplastic lesions which responded in a positive manner to a high calcium diet, and that these lesions had an elevated level of MAPK expression.

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ACF	aberrant crypt foci
AIN	American Institute of Nutrition
AOM	azoxymethane
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
BSA	bovine serum albumin
Ca	calcium
$CaCO_3$	calcium carbonate
CO_2	carbon dioxide
DAG	diacyl glycerol
DCC	deleted in colon carcinoma
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGFR-TK	epidermal growth factor receptor associated with TK
IP_3	inositol tri-phosphate
I-R	insulin receptor
LI	labeling index
MAPK	mitogen activated protein kinase
MBP	myelin basic protein
ODC	ornithine decarboxylase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen

PDGF-R	platelet derived growth factor receptor
PIP_2	phophatidyl inositol bisphosphate
PKC	protein kinase C
P-tyr	phospho-tyrosine
SD	standard deviation
SDA	seventh day adventists
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween 20
TCA	trichloroacetic acid
TI	tumor incidence
TK	tyrosine kinase

Chapter 1

Introduction

1. Introduction

In healthy eukaryotic cells, damage to the DNA contained within the cells can initiate a process known as carcinogenesis. Current research suggests carcinogenesis as a multistage process. Chemical induction (by means of a carcinogen) of carcinogenesis can trigger a sequence of events, some of which are critical to the initiation and establishment of tumor phenotype. Other events that occur in the process may simply be related to the toxic effect of the carcinogen and/or representative of the response of normal tissue to the presence of tumors. To date many of the studies that have examined colon carcinogenesis are fragmented in nature and do not take a stepwise approach to studying the disease. In this regard, it is essential to achieve a comprehensive understanding of the events that are initiated after chemical induction of colon carcinogenesis, and the events occurring in the tumors and colonic mucosa several weeks after the process has begun. Thus, the main objective of this research was to explore and examine the changes in mitogen activated protein kinases (MAPKs) soon after carcinogen exposure in colonic tissue and in colonic tumors.

MAPKs are a family of serine/threonine protein kinases that are involved in the regulation of cell proliferation and differentiation (Crews and Erickson, 1993). PKC and TK are two other families of protein kinases that have previously been explored in colon carcinogenesis. Altered expression and activity of both TK and PKC in the carcinogenic process has been documented, although the findings have not always been consistent (Hug & Sarre, 1993; Cantley *et al.*, 1991). MAPKs can be considered to be one of the missing

components that remains relatively unexplored in colon carcinogenesis. A secondary objective of this thesis research was to provide fundamental information related to the involvement of MAPKs in colon carcinogenesis. It is hypothesized that MAPKs play an important role in colon carcinogenesis and up regulation of the expression of MAPKs will provide an additional growth advantage to selected developing preneoplastic lesions.

Two studies were conducted, the first study explored the early events soon after carcinogen exposure, while the second study explored the later stages of carcinogenesis, tumor development and the effects of dietary calcium supplementation. In the first study PKC, TK, PCNA, cell death and MAPK were assessed during the first 5 days following carcinogen exposure. In the second study, MAPK protein expression was examined in normal colonic mucosa and in colonic tumors, with and without dietary calcium supplementation. A high calcium diet, when given in the later stages of colon carcinogenesis, has been shown to enhance the appearance of adenomas (Pence *et al.*, 1995).

Further, it is speculated that a high calcium diet may lead to increased blood and intracellular calcium. These increased calcium levels may, in turn, stimulate the growth of selected preneoplastic lesions by directly activating the kinases, including MAPK (Figures 2.4 and 2.5).

The specific research objectives were:

1) to assess cell proliferation, cell death and enzymatic parameters (MAPK-

ERK 1 and ERK 2, TK - general, and PKC - Ca dependent) in normal and carcinogen treated colonic mucosa in the first 5 days following carcinogen treatment.

2) to assess MAPK (ERK 1 and ERK 2) expression in tumors and normal appearing mucosa as affected by a high calcium diet.

In keeping with the hypothesis and specific objectives, literature pertaining to colon carcinogenesis, biological endpoints, biomarkers in the study of colon carcinogenesis, and relationship between calcium and cancer are reviewed in the following section.

Chapter 2

Literature Review

2.1 Colon Carcinogenesis

2.1.1 Normal Colon Structure

The wall of the colon consists of four types of tissue, the: serosa, muscularis externa, submucosa, and mucosa (Schauf *et al.*, 1990). In terms of colon cancer, the mucosa is most relevant. The mucosa in composed of epithelium cells which form numerous finger-like projections into the lumen. At the base of these projections are the tube-like crypts of Lieberkühn. Each of these crypts is separated by the lamina propria which consists mainly of connective tissue and is supported by the muscularis mucosa (Schauf *et al.*, 1990). The entire mucosal lining is shed every 5-6 days in humans (Schauf *et al.*, 1990) and every 3-4 days in rats (Maskens and Desjardin-Loitus, 1981).

2.1.2 Multistage Process of Carcinogenesis

Carcinogenesis is currently viewed as a multistage process which begins with initial damage to the DNA of a healthy cell. This damage can occur via a chemical carcinogen, radiation or a virus (Harris, 1991). Once damaged there are three possible outcomes for an altered cell: firstly, it can undergo programmed cell death or apoptosis in which the altered cell dies; secondly it can differentiate which in the case of the colon it will eventually be sloughed off and replaced with new cells; or thirdly it can undergo processes which lead to excessive proliferation, clonal expansion and eventually cancer (Greenwald, 1996). Carcinogenesis itself can be divided into three stages: initiation, promotion and

progression each of which consists of many steps.

Initiation involves exposure of a cell to a carcinogen resulting in genetic changes which enable the initiated cell to respond differently to it's environment. These changes offer a selective growth advantage to the initiated cell compared to the normal surrounding cells (Harris, 1991). For example, a high level of extracellular calcium inhibits cell proliferation in normal cells. However, cells which have suffered genetic damage fail to respond to this cue, and continue to proliferate (Buset *et al.*, 1986; Pitot, 1993).

Promotion, the second stage is characterized by proliferation of the initiated cell into identifiable lesions. Promoting agents, such as cholic acid or a high fat diet, although themselves are not carcinogenic are thought to induce further genetic damage by promoting DNA synthesis and cell proliferation of the initiated cells (Weinstein, 1987).

Progression, the third stage consists of continued phenotypic changes in the initiated population, colonal expansion and invasion of the underlying tissue which eventually results in a metastasis (Harris, 1991).

2.1.3 Regulation of the Cell Cycle

Entry into the cell cycle in which a cell enlarges, replicates its' DNA and eventually divides is regulated by two main gene classes - tumor suppressor genes and protooncogenes. Protooncogenes encourage cell proliferation while tumor suppressor

genes inhibit it. When either or both of these gene classes suffer a mutation the result is uncontrolled cell proliferation. Oncogenes, the mutated form of the protooncogenes, contribute to cancer by producing too much stimulatory protein or an overly active form of it. Tumor suppressor genes on the other hand, contribute to cancer when they become inactivated by a mutation, losing their ability to inhibit proliferation or control cell growth (Weinberg, 1996).

2.1.4 Molecular Basis of Colon Cancer

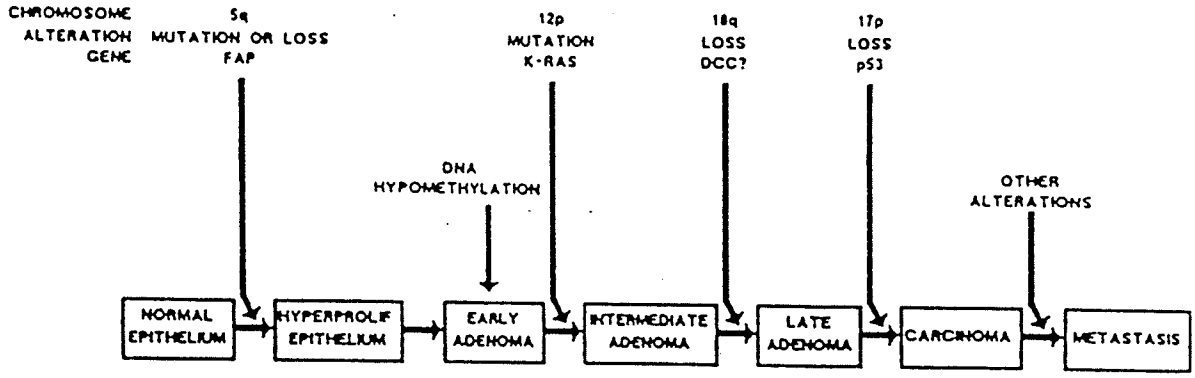
There are at least seven independent mutations which have been detected in a majority of colorectal cancers (Table 2.1). More than 70% of all tumors have mutations in either the adenomatous polyposis coli (APC), deleted in colon carcinoma (DCC), and/or the p53 tumor suppressor genes, while only 2% have mutations in the *neu* and *myc* oncogenes. The fact that each of these mutation is not selected for in every colorectal cancer makes it clear that several distinct pathways can produce this disease (Marx, 1992).

In 1990, Fearon and Vogelstein proposed a genetic model for the development of colorectal cancer (Figure 2.1). This model hypothesizes that in order for a malignant tumor to form, mutations must occur in at least 5 different genes. It also correlates these genetic alterations with various phenotypic changes which occur in the development of the malignant tumor. Fearon and Volgelstein also suggest that it is the accumulation of

Table 2.1: Some mutations detected in colorectal cancer cells (modified from Marx,1992)

Gene	Tumors with Mutations %	Class
K-ras	~50	oncogene
neu	2	oncogene
myc	2	oncogene
APC	>70	tumor suppressor gene
DCC	>70	tumor suppressor gene
p53	>70	tumor suppressor gene
HNPCC	~15	tumor suppressor gene

Figure 2.1: The proposed genetic model for the development of colorectal cancer. The development of a malignant tumor from normal epithelium occurs through mutations in a rate-limiting oncogene and several tumor suppressor genes. These mutations have also been correlated with various phenotypic changes which occur during tumor development (Fearon and Vogelstein, 1990)

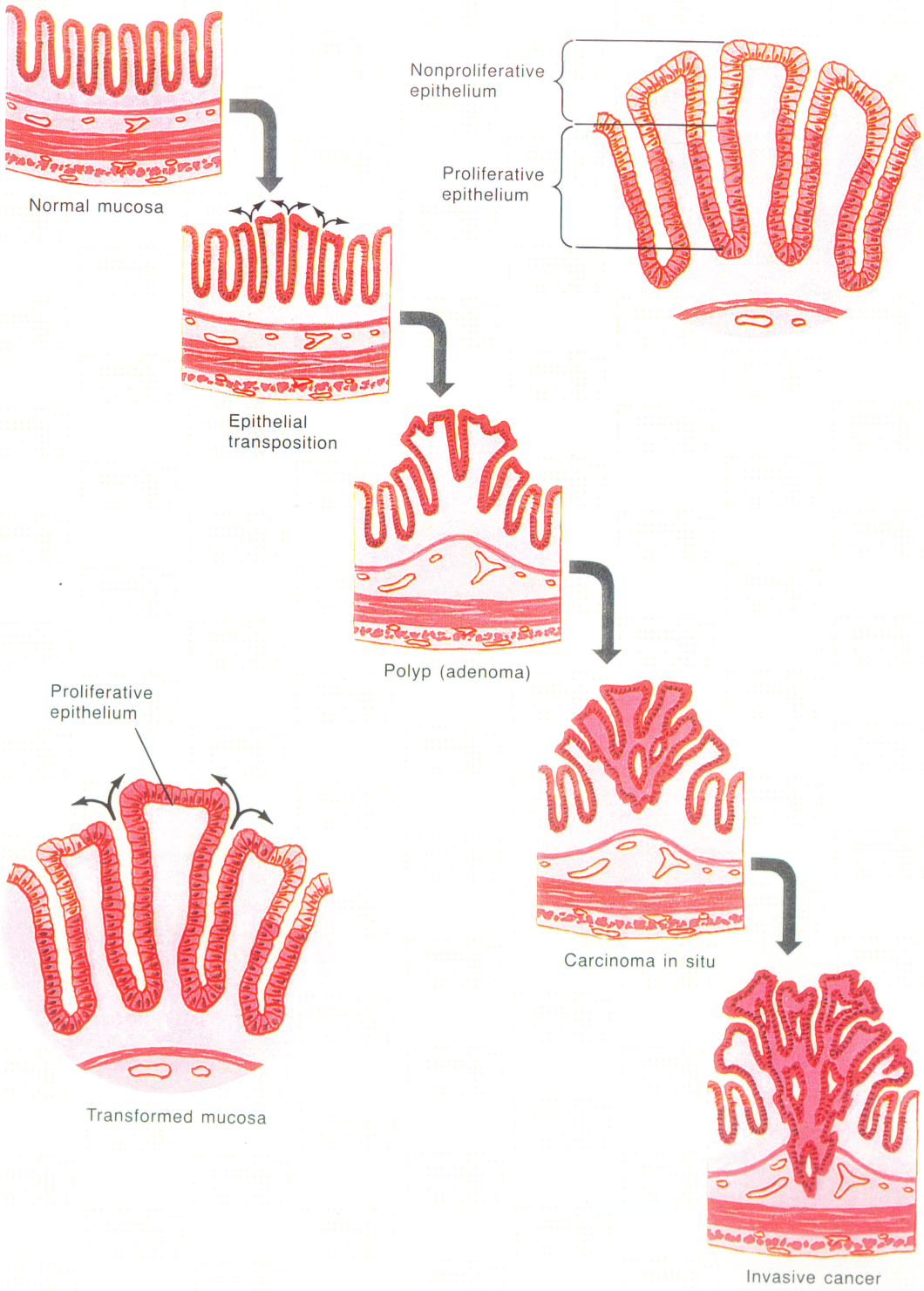


these mutations rather than the order in which they occur, which determines the development of the tumor.

2.1.5 Histogenesis of Colon Cancer

At present, the polyp-cancer sequence (Figure 2.2) for the development of colon cancer is well accepted and is based on the assumption that tumors arise from a single crypt. In the normal colonic mucosa, the crypts are lined with proliferating epithelium in the bottom two thirds of the crypt, while the upper third of the crypts are lined with non-proliferating epithelium. Cancer is initiated in a single crypt when one of these epithelial cells undergoes a mutation, causing the proliferating zone to move towards the surface (Fry *et al.* 1989). These proliferating cells begin to accumulate as they fail to respond to cues for apoptosis and are therefore not exfoliated. This condition is termed hyperplasia (Weinberg, 1996). In addition to this excessive proliferation, when one of these cells undergoes another mutation the resulting cells are also abnormal in both shape and orientation (dysplasia). As the cells acquire more and more mutations, the degree of dysplasia increases as the cycle proceeds in the polyp-cancer sequence. Invasive cancer or malignant cancer occurs when the tumor undergoes genetic changes which enable it to invade the underlying tissue. Once the tumor invades this tissue it can enter into the blood or lymphatic systems and metastasize elsewhere in the body (Fry *et al.*, 1989; Weinberg,

Figure 2.2: The proposed polyp-cancer sequence. Cancer is initiated in a single crypt and with time this crypt becomes morphologically abnormal. Some of the crypts eventually develop into adenomas and adenocarcinomas (Fry *et al.*, 1989)



1996). It is important to note that not all initiated cells will develop into a tumor, and that these mutations usually occur over several decades.

2.2 Biological Endpoints

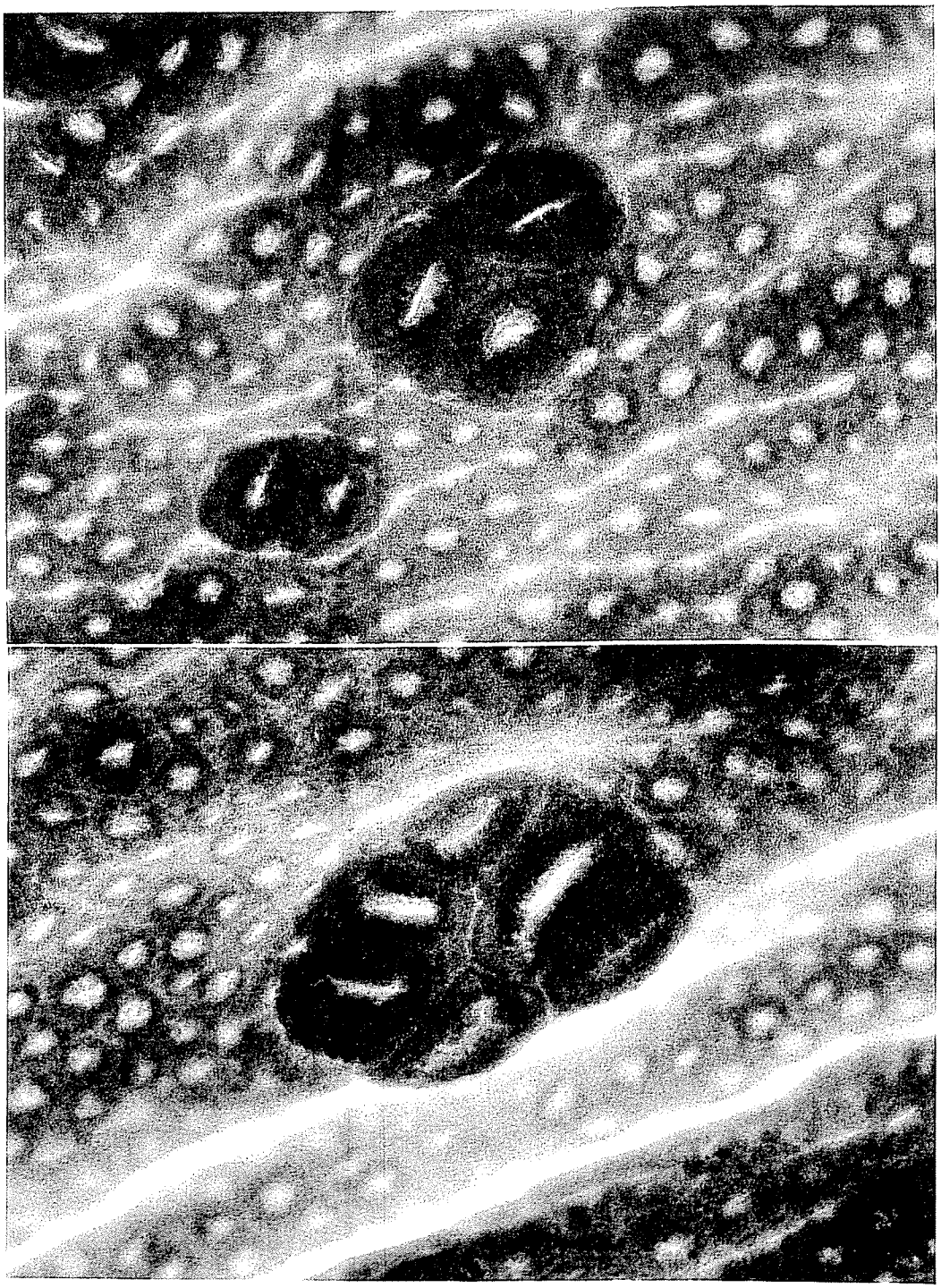
2.2.1 Tumors

Tumors are the most common endpoints used in the study of cancer, as they represent the disease itself. However, it is not possible to assess the ability of various agents in modulating the disease process, using tumors as the only biological marker. In addition, tumor development is a lengthy process which can be quite costly (Bird *et al.*, 1989)

2.2.2 Aberrant Crypt Foci (ACF)

ACF were first identified in 1987 in the colonic mucosa of rats treated with a carcinogen (Bird, 1987). ACF are believed to arise from a single crypt, and are characterized by their thickened epithelial cell walls, along with an increase in crypt height and width compared to normal crypts (Figure 2.3). ACF are more prominent in the proximal colon, and crypt multiplicity has been shown to increase with time (Bird, 1995). Crypt multiplicity is a foci with an increased number of crypts. In general, research substantiates the claim that ACF are preneoplastic lesions (Bird, 1995). The benefits of using ACF as endpoints include; 1) they are precursors of the disease, 2) they change with

Figure 2.3: Topographical view of ACF at various growth stages. A) Two foci with 2 to 3 crypts respectively. B) One focus with six crypts.



time, enabling the researcher to assess them at various stages of the disease, 3) they develop in as little as 2 weeks, thereby decreasing experimental time and cost (Bird *et al.*, 1989). The enumeration of ACF and their growth features is successfully used to study precancerous stages and their growth regulation. The ACF system also allows identification of cancer promoting or preventative agents, which are capable of regulating preneoplastic lesions with a specific geno- and phenotype (Bird, 1995). Although the use of biological endpoints is valuable, it may be more insightful to use intermediate end points to investigate the early signs of the disease. Intermediary end points might also aid in prevention and treatment.

2.3 Biomarkers in the Study of Colon Carcinogenesis

2.3.1 Biomarkers

Biomarkers or risk markers are a group of intermediate end points used to study the effect of various agents on the development of colon carcinogenesis. Biomarkers presently used in the study of colon cancer include cell proliferation, oncogene expression and various enzymatic changes (Lippman *et al.*, 1990, Cooper, 1992). Further research is still required to determine the validity of these intermediate changes in the carcinogenic process. It should be noted that many of these endpoints, with the exception of mutations, are not specific to the carcinogenic process and may not determine the degree of risk harbored by an organ.

2.3.2 Cell Proliferation

Cell proliferation is one the most commonly used risk markers in the study of colon cancer. Increased cell proliferation has been seen throughout the colon in carcinogen treated rodents (Chan *et al.*, 1976), in patients with adenomas or adenocarcinomas (Lipkin, 1983), and in patients who are at high risk for developing colon cancer, such as those with ulcerative colitis (Biasco *et al.*, 1992). However, the value of cell proliferation as a risk marker remains to be established. Several studies have shown that altered cell proliferation does not correlate with tumor development (Karkare *et al.*, 1991, Cameron *et al.*, 1990). Further research is needed to determine the true value of cell proliferation as a risk marker for carcinogenesis. Cell proliferation is a term which encompasses several steps and many regulators. A number of endpoints are used which represent different stages of the cell cycle. The complexity of the cell cycle makes it difficult to determine whether the assessment of the cells in the S-phase actually represent the number of cycling cells. Similarly, quantification of cells expressing the presence of different cyclins may not correctly reflect the number of mitotic events (Sutherland and Bird, 1994).

2.3.3 Signal Transduction

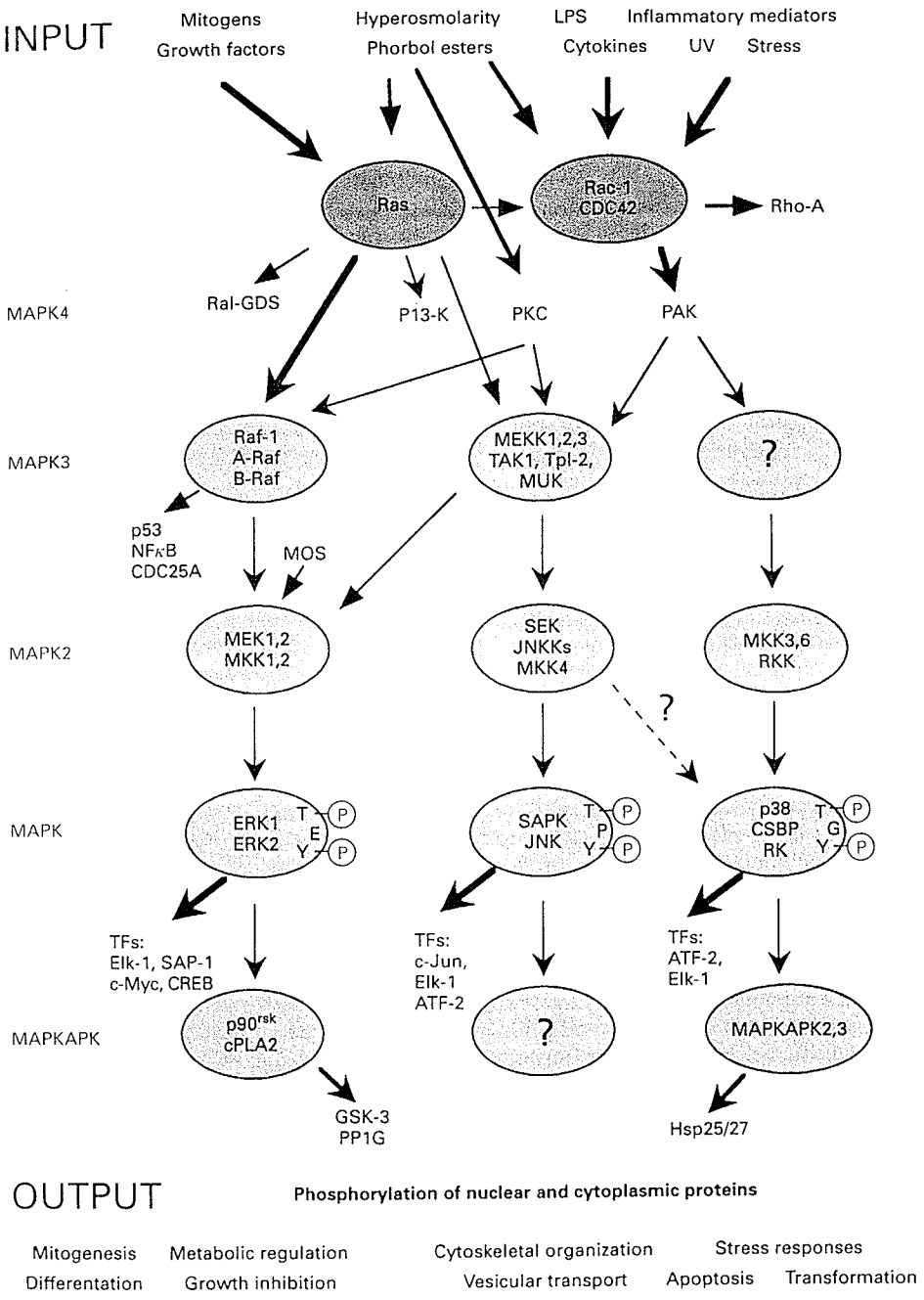
The complex multi-cellular eukaryotic organism requires a highly ordered system of regulation in order for normal cell growth and differentiation to occur properly. A cell

responds to its' environment when a ligand (eg. hormone) binds to a receptor on the cell's surface. The signal travels through the cell by a series of phosphorylation/dephosphorylations of intermediate proteins (effector and/or second messengers) until it reaches it's target protein (Hug and Sarre, 1993). Targets for signal transduction include other cytoplasmic proteins and/or nuclear targets which can ultimately affect gene expression. The majority of the phosphorylations of proteins occur on serine, threonine and tyrosine residues (Bishop, 1991). Protein kinase C (PKC) and mitogen activated protein kinases (MAPK) are two kinase families which phosphorylate serine and threonine residues, while tyrosine kinases (TK) are a family of kinases which phosphorylate the tyrosine residues of it's substrates (Hug and Sarre, 1993; Bishop, 1991). MAPK, PKC and TK families of kinases are inter-related and are implicated in cancer carcinogenesis. Thus, each is described in more detail in the following section.

2.3.3.1 Mitogen-Activated Protein Kinases (MAPK)

MAPKs are an integral part of the *Ras*-mediated signal transduction pathway, and are involved in the regulation of cell proliferation and differentiation (Crews and Erikson, 1993). The general activation pathway for MAPK is through Ras→Raf→MEK→MAPK and is outlined in Figure 2.4 , along with two other related pathways (Denhardt, 1996). These related pathways function more or less independently of the Ras pathway, however some cross-talk does exist. The MAPK family has been highly conserved throughout

Figure 2.4: Signal transduction pathways involving the MAPK family of protein kinases (Denhardt, 1996).



evolution and includes the ERKs, JNKs/SAPKs and the p38/RK MAP kinases (Denhardt, 1996). Various signals preferentially activate different MAPK pathways. ERK1/ERK2, which were studied in this research, are preferentially activated by mitogens and growth factors, whereas SAPK/JNK and/or p38/RK are activated by inflammatory cytokines and various forms of stress (Denhardt, 1996). MAPKs are serine/threonine protein kinases which are identified by their Thr-Xaa- Tyr (where Xaa = Glu, Pro or Gly) motif within the protein kinases subdomain VIII (Davis, 1993; Nishida & Gotoh, 1993). Complete activation of the MAPKs requires phosphorylation on both the Thr and Tyr residues within the TEY motif. MAPKs are generally located in the cytoplasm, but once activated translocate to the nucleus (Davis, 1993; Nishda & Gotoh, 1993). Cellular targets for MAPKs generally contain a minimal consensus sequence which consists of serine and threonine residues directly followed by one or more proline residues (Cobb *et al.*, 1991; Nishida & Gotoh, 1993). MAPK targets include other protein kinases and phosphatases located in the cytosol, nuclear targets such as transcription factors and/or serum response elements and membrane targets such as the Epidermal Growth Factor (EGF) receptor (Davis, 1993). Regulation of MAPK generally occurs in one of two ways; 1) through phosphorylation of upstream signaling components by downstream kinases (negative feedback) and/or 2) by dephosphorylation of key phosphotyrosines by phosphotyrosine phosphatases (Denhardt, 1996).

Point mutations in Ras have been found in high frequency in tumors from various

cancers including colon cancer implicating the involvement of the Ras pathway in cancer development (Bos *et al.*, 1987). However, limited research has been performed to investigate the role of MAPK in cancer. MAPK has been reported to be down regulated in the membranous fractions of both gastric (Atten *et al.*, 1995) and colon adenocarcinomas (Attar *et al.*, 1996). However, an increase in MAPK activity was seen in renal tumors (Oka *et al.*, 1995). Further research is required to determine the precise role of MAPK in the development of colon carcinogenesis. Investigations pertaining to the role of MAPKs in the stepwise development of colon cancer starting from primal preneoplastic lesions to more advanced stages are required. A number of studies have suggested that the physiological conditions under which preneoplatic lesions are initiated may determine the geno- and phenotype of the preneoplastic lesions (Good *et al.*, 1998). Enhanced proliferation predisposes a tissue to develop cancer rapidly. A recent study clearly documents that early events, especially those leading to enhanced cell proliferation are critical in determining tumor outcome (Lasko *et al.*, 1999). In this regard it is pivotal to further define the role MAPKs in the genesis of colon cancer.

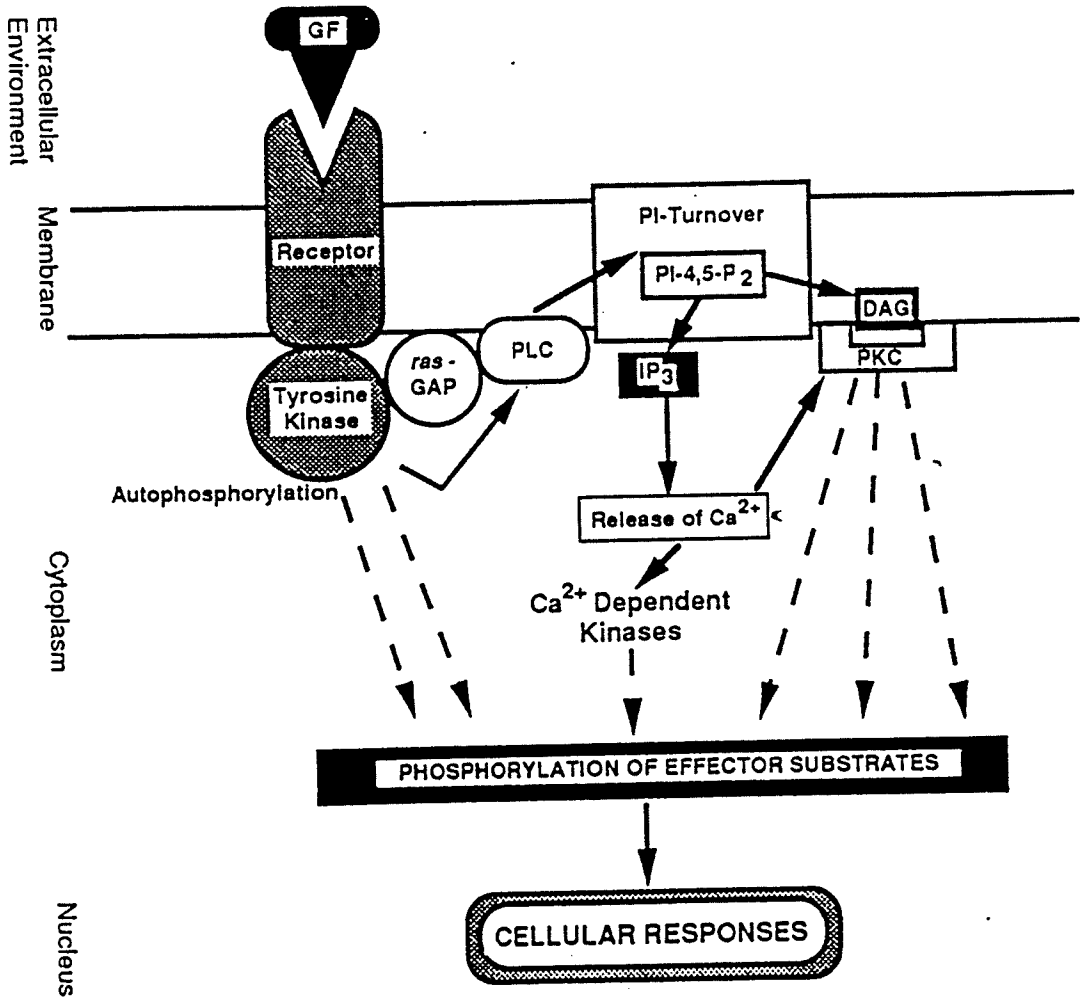
2.3.3.2 Protein Kinase C (PKC)

The PKC family of isozymes consists of twelve different isozymes and can be separated into three distinct groups based on their structure (Dekker & Parker, 1994). The general structure of the PKC enzyme is a polypeptide chain which contains both

regulatory and catalytic domains. Variances in structure among the groups results due to changes in the regulatory domains (Hug & Sarre, 1993). The first and most studied group are the classical PKCs (cPKC) which consist of four isozymes α, BI, BII and γ. These isozymes contain all the regulatory domains, and are activated by calcium, diacylglycerol (DAG) and phorbol esters (Nishizuka, 1992; Sando *et al.*, 1992). The second group, the novel PKCs (nPKC) which consist of δ,ε and θ are calcium independent as they lack the regulatory domain which contains the calcium binding site (Nishizuka, 1992; Sando *et al.*, 1992). The third group of isozymes are referred to as the atypical PKCs and include ξ, ι, λ, μ and η. These isozymes are calcium, DAG and phorbol ester independent as they lack all or part of these regulatory domains (Hug & Sarre, 1993; Nishizuka, 1992; Sando *et al.*, 1992).

PKCs are mainly located in the cytosol and once activated translocate to the membrane. However, the intracellular distribution of the various isozymes varies between tissues and suggests their roles may be tissue specific (Nishizuka, 1992; Houslay, 1991). PKCs are generally thought to be activated when a growth factor binds to its' receptor activating the integral tyrosine kinase that in turn activates the phospholipase C (PLC) enzyme. The PLC enzyme is responsible for cleaving phosphatidyl inositol bisphosphate (PIP_2) into inositol tri-phosphate (IP_3) and DAG. The increase in IP_3 facilitates the release of Ca^{2+} from intracellular stores. The resulting increase in Ca^{2+} and DAG are believed to result in the activation and translocation of PKC to the membrane (Hug & Sarre, 1993; Nishizuka, 1992; Figure 2.5).

Figure 2.5: Proposed model for PKC activation (Radinsky, 1993)



PKC has numerous substrates throughout the cell and these can be divided into three main categories. These include proteins which are involved in; 1) signal transduction and PKC activation (EGF receptor, Ras, GAP), 2) metabolic pathways and 3) regulation of gene expression (Hug & Sarre, 1993).

The fact that PKC can be activated by phorbol esters which are known tumor promoters leads to the investigation of the possible role of PKC in cancer. The involvement of PKC in various cancers has been substantiated, including its role in colon cancer (Blobe *et al.*, 1994). Numerous studies have been performed in tumors from humans (Attar *et al.*, 1996; Levy *et al.*, 1993; Kopp *et al.*, 1991) and in rats (Craven & DeRubertis, 1992; Nelson & Holian, 1991) which found PKC was down regulated in tumors as compared to normal and adjacent mucosa. It has also been proposed that this prolonged down regulation may have resulted from an initially active state, and that an increase in PKC activity may in fact indicate a previously inactive state (Blobe *et al.*, 1994; Craven & DeRubertis, 1992).

Although it has been shown that various PKCs are altered in cancer, further research is required to determine the precise role of the enzyme in the carcinogenic process.

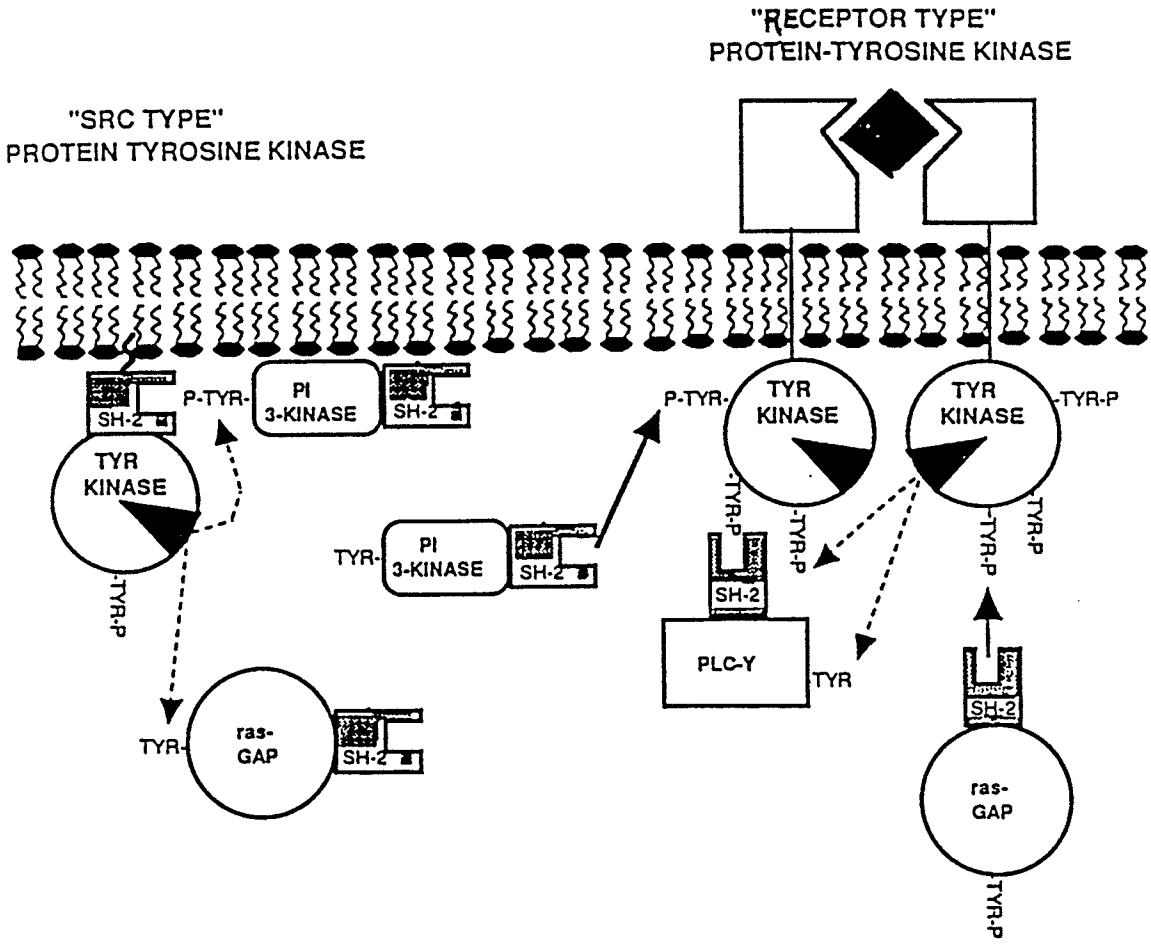
2.3.3.3 Tyrosine Kinases (TK)

The tyrosine kinase family of enzymes is divided into two groups; the

transmembrane receptor family (non-SRC) and the cytosolic non-receptor family (SRC; Figure 2.6; Cantley *et al.*, 1991). The non-SRC family of enzymes can be subdivided into three receptor groups; the epidermal growth factor receptors (EGFR-TK); the platelet derived growth factor receptor (PDGF-R); and the insulin receptor (I-R; Yarden & Ullrich, 1988). In general, the receptors consist of; 1) an extracellular ligand binding domain, to which a growth factor mitogen can bind, 2) a transmembrane domain, which serves to anchor the receptor and provide a link to the intracellular compartment, 3) a cytoplasmic domain which contains a tyrosine kinase catalytic domain. The TK catalytic domain contains a conserved consensus sequence that functions as part of the binding site for ATP (Ullrich & Schlessinger, 1990). Receptor tyrosine kinases are activated when a ligand binds to the receptor causing a conformational change in the extracellular domain, and results in receptor oligomerization. This oligomerization of the receptor aids in the stabilization of cytoplasmic interactions, which ultimately leads to increased tyrosine kinase activity through molecular interactions (Ullrich & Schlessinger, 1990). Once phosphorylated the tyrosine domains can act as binding sites for cytoplasmic signaling proteins which contain SH-2/SH-3 (non-catalytic SRC homology regions) domains (Koch *et al.*, 1991).

The SRC family of enzymes consists of $p60^{c\text{-}src}$ (SRC), $p61^{c\text{-}yes}$ (YES), $p56^{lck}$ (LCK), $p59^{fyn}$ (FYN), $p56^{lyn}$ (LYN), $p55^{c\text{-}fgr}$ (FGR) and $p55^{blk}$ (BLK) (Park *et al.*, 1993). SRC kinases are believed to be activated by growth factors and other cellular activators

Figure 2.6: SRC and non-SRC tyrosine kinase and their potential cellular targets (Cantley *et al.*, 1991).



including PDGF, GTP-γ-s and activators of PKC. SRC kinases are also known to directly associate with the cytoplasmic domain of transmembrane receptors. The best known example of SRC kinases is $pp60^{c\text{-}src}$ (SRC). SRC is phosphorylated on the Tyr 527 residue of the carboxy terminal, and it also contains a SH-2 homology region in it's amino end. When the Tyr 527 residue is phosphorylated, it folds into the SH-2 domain of the same molecule inhibiting protein tyrosine kinase activity. SRC is believed to be activated by dephosphorylation of the Tyr 527 residue which prevents it from binding to the SH-2 region, thus enabling this region to interact with its substrates (Cantley *et al.*, 1991). As evident from Figure 2.6, substrates for SRC kinases include; phosphatidylinositol-3-kinase (PI3-kinase), Ptd Ins-specific PLC-γ, RAS/GAP proteins and $pp74^{c\text{-}raf}$ serine/threonine kinase (Koch *et al.*, 1991; Cantley *et al.*, 1991).

Both SRC and non-SRC tyrosine kinases have been implicated in cancer development. General tyrosine kinase activity (SRC and non-SRC combined) has been shown to be elevated in colonic mucosa of carcinogen injected rats (Rao *et al.*, 1993; Singh *et al.*, 1992; Arlow *et al.*, 1989) and in human colonic carcinomas (Sakanoue *et al.*, 1991). In humans elevated levels of SRC and Yes SRC kinases have been reported (Park *et al.*, 1993; Cartwright *et al.*, 1989; Bolen *et al.*, 1987) in colonic adenomas and adenocarcinomas. Reports of elevated SRC in the epithelium of patients with ulcerative colitis (Cartwright *et al.*, 1994) and benign polyps (Cartwright *et al.*, 1990) suggests that SRC kinases may be involved in the early stages of tumor development. EGFR-TK a non-

SRC kinase has also been shown to be elevated in human carcinoma cells (Radinsky *et al.*, 1995).

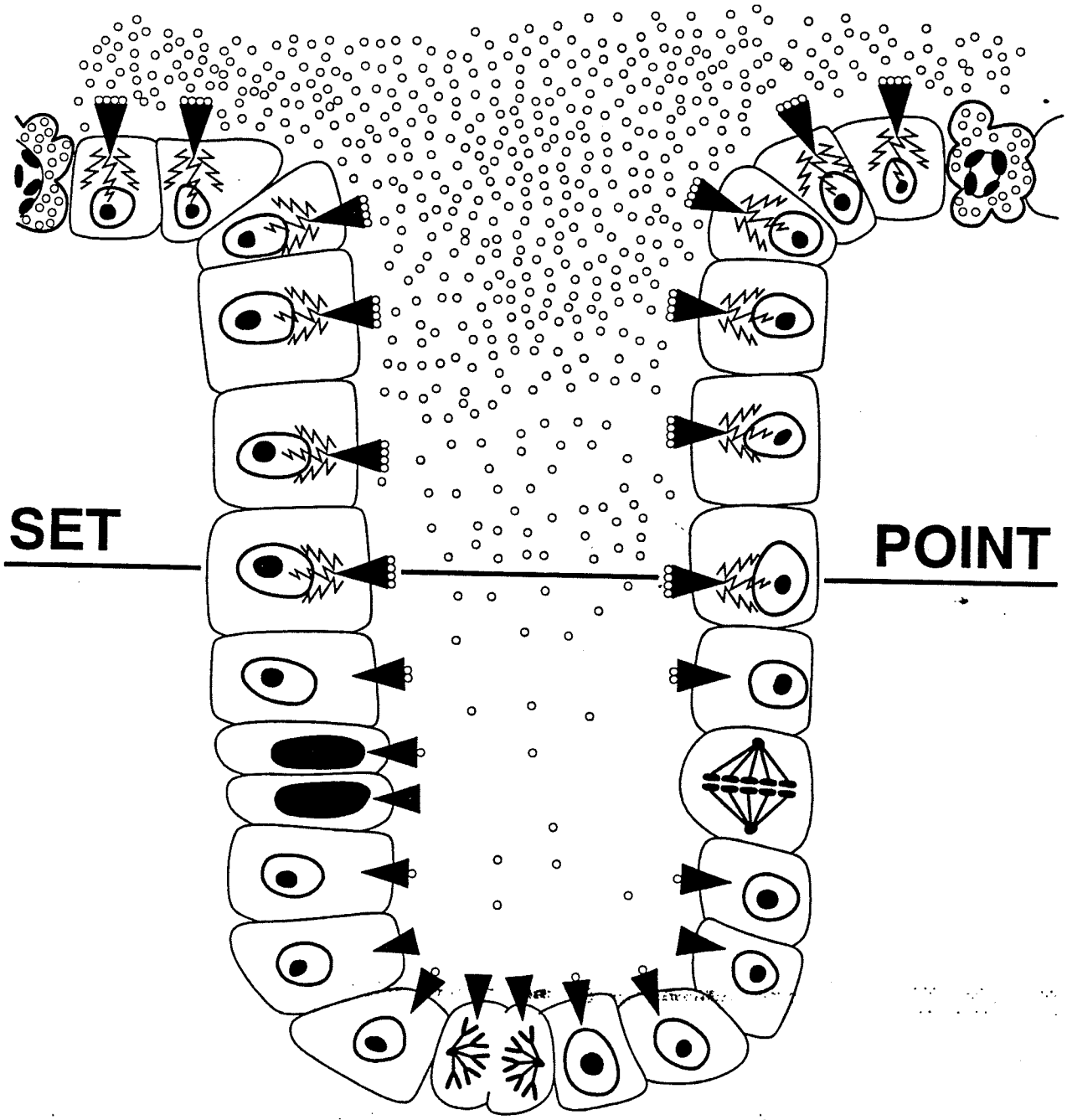
Although tyrosine kinases have been shown to be elevated in early and late stages of cancer development, it has yet to be determined whether or not these altered levels of enzyme activity play a role in the progression of the disease.

2.4 Calcium and Cancer

2.4.1 Growth and Differentiation

Calcium is an essential regulator of a variety of cellular functions, including cell growth and differentiation. Research demonstrates that epithelial cells lining the lumenal surface of the colon are equipped with calcium-sensing receptors on their cellular membrane (Pazionas *et al,* 1994). These colonic enterocytes require about 0.1mM of external calcium in order to proliferate optimally, but at levels between 0.8 mM and 2.2 mM calcium this proliferation is inhibited (Buset *et al,* 1986). This has led to the following proposed model for how external calcium regulates proliferation and induces differentiation (Figure 2.7) Based on the proposed model the concentration of calcium is lowest at the base of the crypt and gradually increases towards the lumen. It is thought that once the calcium concentration reaches the "set point" for the calcium receptor, this results in the activation of a pathway for differentiation. (Whitfield *et al,* 1995).

Figure 2.7: Proposed model for control of cell proliferation and differentiation by external calcium(Whitfield *et al.*, 1995) The concentration of calcium is lowest at the base of the crypt and gradually increases till it reaches the lumen. It is thought that once the calcium concentration reaches a certain "set point" for the calcium receptors, this results in the activation of a pathway for differentiation.



As cells become transformed they reach a point at which they no longer respond to external signals such as calcium (Pitot, 1993). In a study by Buset and colleagues, a high level of external calcium was able to inhibit the proliferation of normal mucosa, but adenomas and carcinomas failed to respond to the inhibitory effect of high external calcium (Buset *et al*, 1986).

2.4.2 Epidemiological Studies

Throughout the years numerous epidemiological studies have examined the relationship between calcium intake and colorectal cancer and is the subject of a recent review (Hyman *et al.*, 1998). One of the first studies looked at Seventh Day Adventists (SDA) due to their low incidence of colon cancer. A positive correlation was observed between greater consumption of milk and a reduced risk of colon cancer as compared to controls (Phillips, 1975). Other studies revealed similar results (Garland et al , 1985; Slattery et al, 1988) however, some report no correlation suggesting there may be confounding variables (Heilbrun et al, 1986, Kampman et al, 1994). The SDA populations for example, represents a distinct population with similar genetics and dietary practices (Phillips, 1975). Perhaps the decrease incidence for colon cancer is due not only to the higher consumption of milk, but to the lack of alcohol, caffeine or meat in their diets. Therefore, the results of these studies do not conclusively indicate a relationship between increased dietary calcium and a decreased risk for developing colon cancer.

2.4.3 Cell Proliferation in Animal Studies

The prospect of calcium as a chemopreventive agent first gained recognition in 1983 when Wargovich and colleagues reported that calcium was able to alleviate the hyperproliferation induced by intrarectally administered fatty acids (Wargovich *et al*, 1983; Wargovich *et al.*, 1984). The calcium effect was measured by its ability to significantly reduce the number of tritiated labeled cells and mitotic figures per crypt. Further research revealed that the colonic epithelium of animals fed a low calcium diet had higher proliferation with or without chronic exposure to bile acids, than those fed a high calcium diet (Bird, 1986, Bird *et al.*, 1986).

The ability of high dietary calcium to alleviate hyperproliferation is not limited to the colon. Indeed, increased calcium has been shown to decrease the hyperproliferation promoted by a high fat diet, in murine mammary epithelial (Jacobson *et al.*, 1989).

2.4.4 Tumorogenesis in Animal Studies

The results of the animal studies which looked at calcium as a modulator of colon carcinogenesis are conflicting. While some studies have shown a possible role for calcium as a chemopreventive agent, others do not substantiate this role and some even suggest that a high calcium diet may enhance tumor development. Discrepancies between studies may be due in part to variations in protocols. Study protocols differ in the number of carcinogen injections (1-10), dose of the carcinogen (1.5 mg - 200 mg/kg), time of

intervention (1-8 weeks before, concurrent, 1-38 weeks after carcinogen injection), and the level of calcium given (0.04% - 2.0%). These differences make it difficult to compare studies directly.

Several studies have shown a reduction in tumor incidence (TI) when higher amounts of calcium were fed. The TI was reduced when animals were fed three times the recommended amount of calcium in the presence of a high fat diet (Skrypec and Bursey, 1988). This was also seen in a study by Pence and Buddingh (1988), however in the presence of a low fat diet TI was unaltered. The TI was found to be higher in a low calcium diet in the presence of high fat, but the significance was lost in the presence of a low fat diet. However, adenocarcinomas were significantly reduced in the higher calcium diet in the presence of both high and low fat (Wargovich *et al.*, 1990)

Not all studies have seen a reduction in TI. In a study by Nelson *et al.* (1987), supplementation with either milk or calcium failed to reduce the TI as compared to controls. No effect on TI was seen whether calcium was administered before or simultaneously with the first carcinogen dose. However, the high dietary calcium groups had a significant reduction in the number of invasive carcinomas (McSherry *et al.*, 1989). In a study by Pence *et al.* (1995), calcium fed for 38 weeks following carcinogen injection and then discontinued had no effect. However, calcium introduced 38 weeks into the study and fed for 4 weeks also did not affect TI, but tumor burden was increased compared to the controls.

2.4.5 Clinical Trials

In 1985, Lipkin and Newmark were the first to attempt "the calcium intervention strategy" in humans. Patients who were at high risk for the development of colon cancer were given 1250 mg of calcium per day in the form of $CaCO_3$. After three months patients showed a significant decrease in rectal proliferation. Since then, a number of similar clinical trials have been performed in high risk and adenoma patients. Favorable results were also seen in adenoma patients (Rozen *et al.*, 1989; Wargovich *et al.*, 1992), intestinal bypass patients (Steinback *et al.*, 1994), first degree relative of colorectal patients (Rozen *et al.*, 1989) and patients at high risk for developing hereditary nonpolyposis colorectal cancer (Cats *et al.*, 1995).

Conversely, calcium supplementation failed to decrease hyperproliferation in first degree relatives of colorectal patients (Gregoire *et al.*, 1989), patients with familial adenomatous polyposis (Stern *et al.*, 1990) and patients with sporadic adenomas (Baron *et al.*, 1995; Bostick *et al.*, 1995; Bostick *et al.*, 1993). Although calcium supplementation had no effect on proliferation in the study by Bostick *et al.* (1995), it normalized the distribution of the proliferating cells in the colonic crypt.

2.4.6 Mechanisms of Calcium Inhibition

In 1984 Newmark and colleagues were the first to hypothesize the mechanism of inhibition of calcium supplementation on colonic tumors. Their hypothesis was based on

the assumption that dietary fat promotes colon cancer by increasing the concentration of free ionized fatty acids and bile acids in the colonic lumen which are irritating to the colonic epithelium. Calcium was thought to neutralize these acids by forming non-irritating insoluble soaps (Newmark *et al.*, 1984)

Since the "calcium soap" hypothesis, other non-luminal mechanisms have been proposed. Calcium supplementation decreases the activity of enzymes involved in proliferation and signal transduction. Specifically, calcium supplementation decreased the activity of ornithine decarboxylase (ODC; Baer and Wargovich, 1989; Arlow *et al.*, 1989; Lans *et al.*, 1991), TK; (Arlow *et al.*, 1989) and PKC (Pence *et al.*, 1992). Calcium supplementation was also found to prevent K-*ras* mutations which were found in one third of all tumors in the non-supplemented group (Llor *et al.*, 1991).

Calcium's ability to reduce proliferation and TI is likely due to a combination of both luminal and non-luminal mechanisms. More research is required to elucidate calcium's chemopreventative mechanism.

Chapter 3

Methods and Materials

3. Methods and Materials

3.1 Chemicals

All chemicals were purchased from Sigma Chemical Co., Mississauga, Ontario unless otherwise stated. Chemical composition of all buffers, gels, and cocktails are listed in the Chapter 7.

3.2 Animals and Animal Care

Male weanling Sprague Dawley rats (Central Breeding, University of Manitoba, Winnipeg, Manitoba) were used. Animals were housed in stainless steel wire cages with sawdust bedding and kept in a controlled environment consisting of a 12 hour light-dark cycle, temperature at 22°C and humidity at 55%. Animals were given standard laboratory chow and water ad libitum during the two week acclimatization period. All animals were cared for according to the guidelines of the Canadian Council on Animal Care (University of Manitoba Animal Care Comittee).

3.3 Diet

The diet was based on a semi-synthetic AIN-93 (American Institute of Nutrition) standard diet (Table 3.1) or a modified version (Table 3.2; Reeves, *et al*, 1993).

3.4 Study I Design

Eighty weanling Sprague Dawley (~300g) rats were used. After two weeks

Table 3.1: Composition of the AIN-93M semi-synthetic diet for the maintenance of adult rodents

Ingredient	**g/kg diet**
Corn starch	465.692
Casein (≥85% protein)	140.000
Dextrose	255.000
Soybean oil	40.000
Fiber	50.000
Mineral mix (AIN-93M-MX)*	35.000
Vitamin mix (AIN-93-VX)*	10.000
Choline bitartrate	2.500
L-Cystine	1.800

*refer to the Appendix for content

Table 3.2: Composition of the Modified AIN-93M diet

Ingredient	g/Kg diet
Corn starch	337.50
Casien (≥% protein)	230.00
Dextrose	85.00
Soybean oil	5.00
Fiber	59.00
Mineral mix (AIN-93M-MX)*	41.00
Vitamin mix (AIN-93-VX)*	11.50
Choline bitartrate	2.30
L-Cystine	3.00
Beef tallow	180.00
Calcium carbonate	37.50

*refer to the Appendix for content

acclimatization on standard laboratory chow, the animals were housed 2-3 per cage and switched to the AIN-93 semi-synthetic diet. After 1 week, 45 of the 80 animals were injected sub-cutaneously, in the lower thigh, with azoxymethane (AOM) at a dose of 15 mg/kg body weight. Nine injected and 7 control animals were then killed every 24 hours for 5 days. All animals were killed by CO_2 asphyxiation. Colons were excised, flushed with cold PBS, slit from the caecum to the anus and placed on a cooled 4°C surface. A 1 cm^2 section was taken from the rectal and mid regions of the colon and fixed in 70% ethanol for sectioning at a later date. The remaining colonic mucosa was separated via region (distal or proximal) and scraped. The mucosal scrapings were frozen immediately in liquid nitrogen and stored at -80°C until separated into cytosolic and membranous fractions.

3.5 Study II Design

Twenty-five weanling Sprague Dawley rats (~300 g) were used. After two weeks acclimatization, 20 of the 25 animals were injected with AOM (15mg/kg body weight) once a week for three weeks. All animals were then fed a standard laboratory chow for 25 weeks at which time they were switched to a modified AIN-93 diet which contained either a normal level of calcium (0.5%) or a high level of calcium (2.0%) (Table 3.2). Animals were fed this modified diet for three weeks. All animals were killed by CO_2 asphyxiation. Colons were removed, flushed with cold PBS, slit from the caecum to the anus and placed

on a cooled 4^0C surface. Tumors were excised and size and location recorded. The remaining mucosa was separated via region (distal or prximal) and scraped. All samples were frozen immediately in liquid nitrogen and stored at -80^0C until used.

3.6 Separation of Colonic Cytosolic and Membranous Fractions

Approximately 0.3 grams of frozen (-80°C) colonic tissue was placed in 3 mL of ice cold cytosolic buffer. The tissue was then homogenized with a Polytron (PT 1020-350D) and centrifuged at 100,000 x *g* in an ultra centrifuge (Beckman L5-50B) for 1 hour at 4°C. The supernatant (cytosolic fraction) was removed and aliquoted (600μL). The pellet was removed and placed in 3 mL of ice-cold membranous buffer. The samples were then homogenized with a Polytron (PT 1020-350D) and centrifuged for 30 minutes at 100,000 x *g* (Beckman L5-50B ultracentrifuge) at 4°C. The supernatant (membranous fraction) was removed and aliquoted (600 μL). Both membranous and cytosolic fractions were stored at -80°C until required for the kinase assays or Western blots.

3.7 Whole Cell Lysate

Approximately 0.1g of powdered (by hammer), frozen tissue (tumor or mucosa) was placed in 0.3 mL of ice cold RIPA buffer. The tissue was homogenized and set on ice for 30 minutes. The homogenized tissue was then centrifuged at 15000 x g for 30 minutes at 4^0C. The supernatant was further separated with an additional centrifugation (15000 x

g, 4^{0}C) for 20 minutes. The resultant supernatant was removed and frozen at -80^{0}C until used for Western blot analyses.

3.8 Protein Analysis

Protein concentration was determined using the Coomassie protein assay reagent (Pierce, Rockford, IL.) and the method developed by Bradford in 1976. Bovine serum albumin (BSA) (Pierce, Rockford, IL.) was used as the standard. All samples were performed in duplicate.

3.9 Cell Proliferation

Proliferating cell nuclear antigen (PCNA) expression was determined using an immunohistochemical technique based on the method developed by Richter *et al.*, 1992. Mid and rectal colonic sections previously fixed in 70% ethanol were embedded in paraffin wax, sectioned to a thickness of 5 μm, and longitudinal unstained sections were mounted onto slides (Dept. of Pathology, St. Bonifice Hospital, Winnipeg, MB). Immunohistochemical techniques were carried out utilizing the bulk Histo-Stain SP kit (Zymed, London ,Ontario). Briefly, slide sections were deparaffinized, and blocked for 20 minutes with normal goat serum to prevent non-specific binding. Sections were then incubated with the primary antibody, PCNA (1:40) (Dimension Laboratories Inc., Mississauga, Ontario) for 1 hour. Following this, the sections were incubated with anti-

mouse IgG (antibody bridge) for 20 minutes. The slides were then immersed in 0.06% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.03% H_2O_2 (BDH Inc., Toronto, ON) for 10 minutes to initiate the peroxidase reaction, and then were counter stained with haematoxylin. The slides were then dehydrated and mounted with permount (Fisher Scientific, Ottawa, Ontario). All slides were washed with fresh PBS between each incubation, and all incubations were performed at room temperature in a humid ambient chamber. PCNA labeled cells were classified as those cells which were darkly stained along the length of the crypt. The number and position of each labeled cell was recorded in 10 crypts per colon. The labeling index (LI) was calculated as a percentage of total cells, and is defined by the number of labeled cells per crypt divided by the total number of cells along both sides of the crypt, multiplied by 100.

3.10 Cell Death (Apoptosis)

Apoptotic bodies were recorded on the same slides as the PCNA staining, and were also recorded for 10 crypts per colon. Cells that were stained brown and exhibited karyorhexis (fragmented nucleus) or pyknosis (condensed, glossy nucleus) were said to be apoptotic.

3.11 Detection of Proteins by Western Blotting

The method followed for Western blotting was that initially developed by Laemli in

1970. Equal volumes of protein sample and 2X SDS sample buffer were mixed and boiled for 1 minute at 90°C. Protein samples (5-15 μg of protein) were loaded at a concentration of 1μg/μL on a discontinuous denaturing gel system consisting of a 0.75 mm 5% stacking and 10% separating SDS-PAGE, and ran in 600 mL running buffer at 170V for 1 hour and 15 minutes. The proteins were then transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL), utilizing a BIO-RAD Mini-Protean cell (BIO-RAD, Mississauga, Ontario) containing 600 mL transfer buffer, at 100V for 2 hours. The membranes were blocked for 1 hour at room temperature. The membranes were washed for 30 minutes (4X20 mL) with TBS-T and then probed with the primary antibody overnight at 4°C. The membranes were then washed with TBS-T (4X20 mL) for 30 minutes and probed with the appropriate secondary antibody for 1 hour at room temperature. Membranes were washed for 1 hour (8X20 mL) with TBS-T. The immunoreactivity was detected using an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) and Kodak X-OMATAR film (Eastman Kodak Company, Rochester, NY). Even and efficient transfer of loads was monitored utilizing the India Ink (membrane) and Coomassie blue (gel) staining techniques. The resulting films were scanned using a Reliasys Image Scanner and Photoshop version 2.5 software for Macintosh. The area of each protein band (mm^2) was determined using Image version 1.49 software (error +/-0.05 m^{m2}). All gels ran contained at least one sample which was the same so that a correction factor could be determined and the resulting films for the

same antibody could then be compared. The correction factor was calculated as a percentage of the area determined for the common sample. All samples on that gel were then multiplied by that factor.

3.12 Conditions for various antibodies

3.12.1 MAPK (ERK1-CT)

For the colonic mucosa cytosolic and membranous fractions, 5 μg of protein was loaded for each sample. For tumor whole cell lysate fractions, 10 μg of protein was loaded for each sample. A 3T3 cell lysate (6 μg) was loaded as a positive control. The blocking solution used was 5% skim milk powder in TBS-T. The primary antibody, ERK1-CT (UBI, Lake Placid, NY) and the secondary antibody, rat anti-rabbit horse radish peroxidase conjugated IgG were both diluted 1/1000 in 5% skim milk in TBS-T.

3.12.2 Phospho-tyrosine (P-Tyr)

For the colonic mucosa cytosolic fractions 10 μg of each protein sample was loaded, and 15 μg of protein was loaded for each of the membranous fractions. A 3T3 cell lysate (12 μg) was loaded as a positive control. The blocking solution used was 3% BSA in TBS-T. The primary antibody, P-Tyr (Transduction Labratories, Lexington, Kentucky) and the secondary antibody, rat anti-mouse HRP conjugated IgG were both diluted 1/1000 in 1% BSA.

3.13 MAPK Immunoprecipitation

The immunoprecipitation protocol provided by Upstate Biotechnology Inc. was followed.

Method A: Briefly, the cell lysate was pre-cleared by incubating it with 100 μL of the proteinA agarose bead slurry (UBI, Lake Placid, NY) for 10 minutes at 4°C. The beads were pelleted at 14000 rpm (Beckman GSISR) for 5 seconds and 500 μg of the cell lysate was incubated overnight at 4°C with 4 μg of the ERK1-CT antibody (UBI, Lake Placid, NY). The immune complex was captured by the addition of 100 μL of protein Agarose bead slurry (UBI, Lake Placid, NY) and the solution was incubated at 4°C for 2 hours. The immune complex was pelleted at 14000 rpm (Beckman GSISR) for 5 seconds and washed (3x500 μL) with ice cold PBS. The resulting immune complex was then used immediately in the MAPK assay.

3.14 MAPK Activity Assay

Colonic MAPK activity was measured using the MAPK assay kit from Upstate Biotechnology (Lake Placid, NY). The MAPK assay kit measures the transfer of the γ phosphate group of [γ-^{32}P] ATP to the substrate myelin basic protein (MBP). Briefly, 10 μL of ADB, substrate cocktail, and inhibitor cocktail were added directly to the immuno-complex. Radiolabeled γ-^{32}P ATP (specific activity = 3000 Ci/mmol) was added to the

kit magnesium ATP buffer (500 μM cold ATP, 75mM magnesium chloride in ADB) at a level of 10 μCi/10 μL of buffer. The reaction was started by the addition of 10 μL of the magnesium γ-^{32} P ATP buffer and the reaction mix was allowed to incubate for 20 minutes (10 min for method A) at 30 °C. The reaction mix was microfuged to pellet the beads, and the reaction was stopped by spotting 35 μL of the reaction mix onto P81 paper squares. The filter papers were washed 3 times with 0.75% phosphoric acid for 5 minutes each and then for 5 minutes with acetone (~200 mL). The filter papers were then transferred into scintillation vials containing 10 mL of Cyto-scint (ICN, Montreal, Canada) and counted for 1 min using a LS-6000TA Beckman Scintillation Counter. Duplicates of all samples were performed. The assay was also performed in the absence of the substrate and inhibitor cocktails in order to account for endogenous phosphorylation. The specific activity was expressed as pmol P_i transferred/min./mg protein.

Calculation for MAPK Activity

1. Specific Activity of ATP

   10 μL of ATP mixture contains $5x10^{-9}$ M ATP

2. Activity of Sample

   S=Activity of reaction mixture (with substrate) - Activity of reaction mixture (without substrate)

3. MAPK Activity (U)

U=S/(SA) (20 min) (mg protein)*

*For method A 10 minutes was used.

3.15 PKC Activity Assay

Colonic PKC activity was measured using the PKC assay kit (Amersham Life Sciences, Arlington Heights, IL). The PKC kit measures the transfer of the γ phosphate group of [γ-^{32}P] ATP to a PKC specific substrate. Briefly, equal amounts of lipid buffer, PKC peptide buffer, calcium buffer and DTT buffer were mixed and 25 μL of this component mixture was added to each reaction tube. Twenty-five μL (30-60 μg) of colonic membranous sample was added to each reaction tube. Radiolabeled γ-^{32}P ATP (specific activity=3000Ci/mmol) (NEN, Boston, MA) was added to the kit magnesium ATP buffer (1.2 mM ATP in 30 mM Hepes, 72 mM $MgCl_2$) at a concentration of 0.5 μCi/5 μL of buffer. The reaction was started by the addition of 5 μL of the magnesium γ-^{32}P ATP buffer and the reaction mixture was allowed to incubate for 15 minutes at 37^{0}C. The reaction was halted by the addition of 10 μL of the kit stop reagent (300 mM orthophosphoric acid with carmosine red). The total reaction mixture was then spotted onto P81 filter paper. The filters were then washed with 10% acetic acid (3x10 minutes), transferred into scintillation vials containing 10 mL of Cytoscint (ICN, Montreal, Quebec). Samples were counted for 1 minute using a LS-6000TA Beckman scintillation counter.

Duplicates of all measurements were performed. The assay was also performed in the absence of the PKC peptide buffer in order to account for endogenous phosphorylation. The specific activity was expressed as pmol Pi transferred/min/mg protein.

Calculation for PKC Activity(V):

1. Specific Activity of ATP (SA)

   5 μL of ATP mixture contains $6x10^{-9}$ M ATP

2. Activity of Sample (S)

   S= Activity of reaction mixture (with peptide) - Activity of reaction mixture (without peptide)

3. PKC Activity (V)

V= S/(SA) (15 min) (mg protein)

3.16 TK Activity Assay

TK activity was measured using the method developed by Rao *et al.*, 1993. The method measures the transfer of the γ phosphate group of [γ^{-32}P] ATP to the glutamic acid-tyrosine polymer (poly-glu). Briefly, 25 μL (20-50 μg of protein) of membranous or cytosolic colonic fractions was added to each reaction tube, along with 5 μL poly-glu (final conc. 20 μg/50 μL). Radiolabelled γ^{-32}P ATP (specific activity =3000Ci/mmol) was

added to a reaction mix (50 mM Tris(pH 7.5), 20 mM $MgCl_2$, 0.02% TritonX-100, 50 mM $NaVO_4$ and 50 μM ATP) at a level of 0.5 μCi/20 μL reaction mix. The reaction was started by the addition of 20μLl of the reaction mix containing γ^{-32}P ATP to the sample and substrate mix. The resulting reaction mix was allowed to incubate for 15 minutes at 24°C, and stopped by spotting the mixture on P81 filter paper. The filter papers were then washed with 10% trichloroacetic acid (TCA) with 10 mM sodium pyrophosphate (3x5 min),and ethanol (1x5 min). The filter papers were transferred into scintillation vials containing 10 mL of Cyto-scint (ICN, Montreal, Quebec) and counted for 1 minute using a LS-6000TA Beckman scintillation counter. Duplicates of all measurements were performed. The reaction was also performed in the absence of the poly-glu substrate in order to account for endogenous phosphorylation. The specific activity was expressed as Pi transferred/min/mg protein.

Calculation for TK Activity (W):

1. Specific Activity of ATP (SA)

   20 μL of ATP mixture contains $1x10^{-6}$ M ATP

2. Activity of the Sample (S)

   S= Activity of the reaction mixture(with polymer) - Activity of the reaction mixture (without the polymer)

3. TK Activity (W)

W= S/(SA) (15 min) (mg protein)

3.17. Statistical Analysis

3.17.1 Study I

Statistical analysis of the data was performed using Analysis of Variance (ANOVA) in conjunction with Duncan's Multiple Range Test. All tests were conducted using the SAS Statistical software package (SAS Institute Inc., Cary, N.C.). A p value of ≤ 0.05 was considered significant.

3.17.2 Study II

Statistical analysis of the data was performed using Chi Squared analysis. A p value of < 0.01 was considered significant.

Chapter 4

Results

4. Results

4.1. Cell Proliferation and Apoptosis

4.1.1 PCNA Expression

In the colonic crypts from Sprague-Dawley rats killed 1 to 5 days after injection, mid and rectal sections displayed the same pattern of PCNA expression. Thus, mid and rectal sections were combined and are shown in Figure 4.1. The labeling index was lower on day 1 and day 2 and higher on day 4 and day 5 in colon sections from the injected group as compared to the control group.

4.1.2 Cell Death

Apoptotic bodies were enumerated in the colonic tissue of Sprague-Dawley rats killed 1 to 5 days after injection. Mid and rectal sections showed similar trends and the combined results are depicted in Figure 4.2. The number of apoptotic bodies was found to be higher on day 1 and day 2 in the injected group, and gradually decreased to control levels by day 4.

4.2. Enzymatic Activity

4.2.1 PKC Activity

The PKC activity as determined in the membranous fraction was found to be variable in both control and injected animals. No significant differences were found. On

Figure 4.1 PCNA labeling indices in colonic crypt sections from male Sprague Dawley rats killed 1 to 5 days for both injected and control groups. (n = 7 control n=9 injected, mean ± SEM)

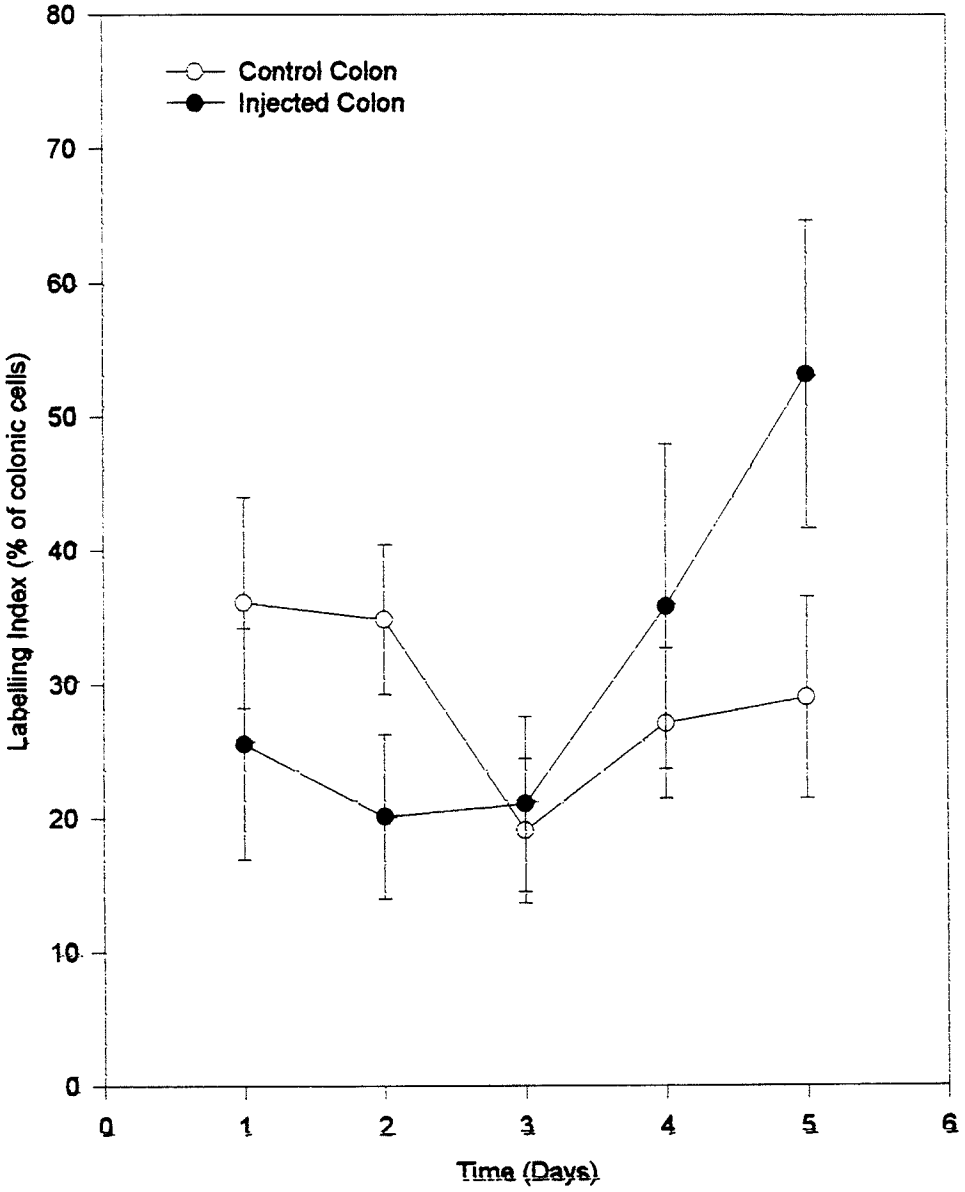
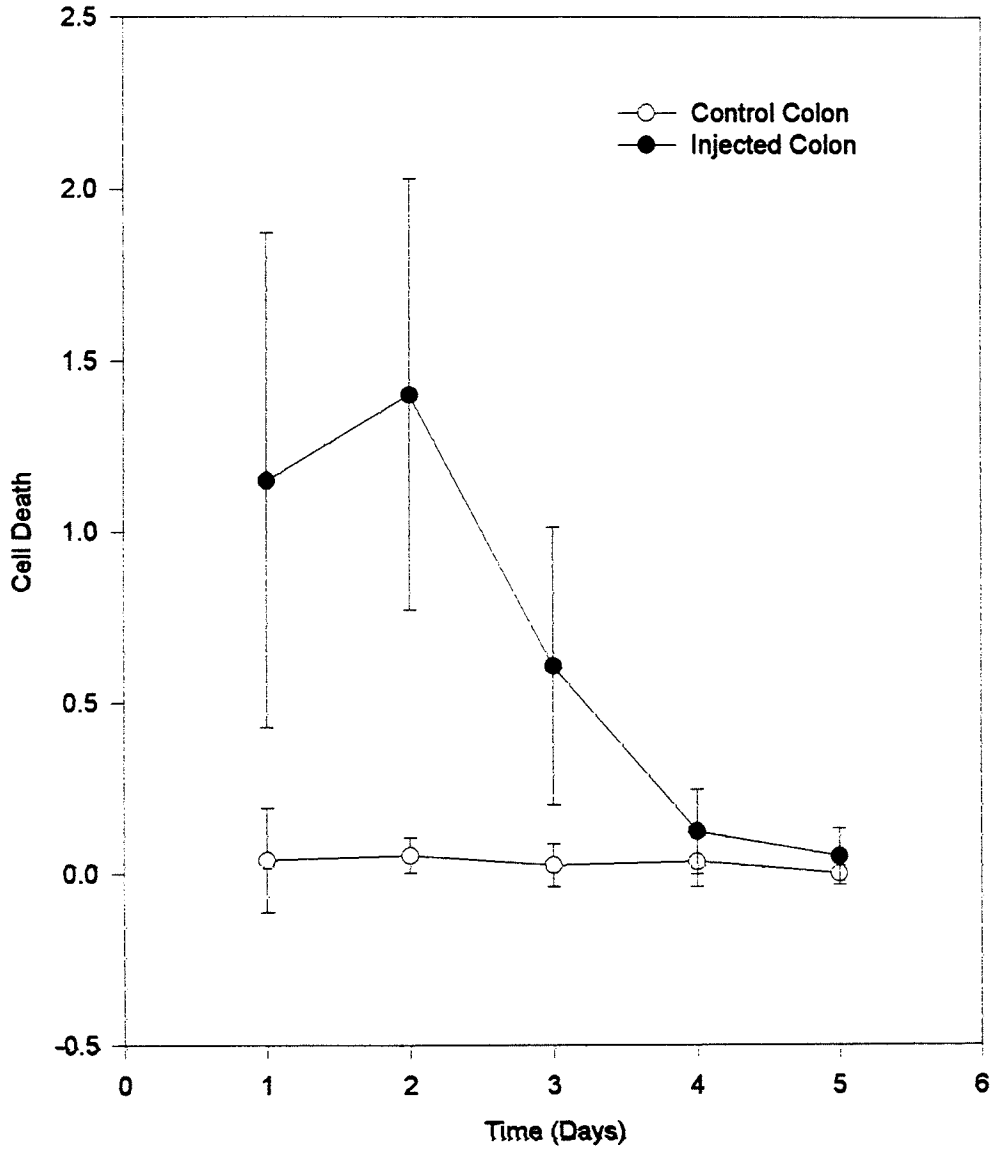


Figure 4.2 Apoptosis in colonic sections from male Sprague Dawley rats killed 1 to 5 days for both injected and control groups. (n = 7 control, n=9 injected, mean ± SEM)



day 1 distal membranous activity (injected group) was lower than the distal control, where as the proximal membranous activity (injected group) was higher than the proximal control. With the exception of day 1, the distal and proximal fractions behaved similarly with respect to PKC activity (Figure 4.3) and therefore the results were combined (Figure 4.4). The membranous PKC activity in the injected group was highest on day 2 and gradually decreased to its lowest value on day 4. The PKC activity then returned to control values by day 5.

4.2.2 TK Activity

TK activity was determined using a method developed by Rao and colleagues in 1993. The cytosolic activity (Figure 4.5) appears to be higher in the control groups on both day 1 and day 5 as compared to the injected groups and both the control and injected groups are higher on day 5 than on day 1. The distal activity appears to be higher on both day 1 and day 5 compared to the proximal activity in both injected and control samples. The membranous activity (Figure 4.6) was higher in the injected groups as compared to the control groups. The activity for the injected group was higher on day 5 as compared to day 1, and this increase in activity was higher in the proximal colon than in the distal colon. Repeating the activity for the distal membranous fractions at a later date resulted in similar activity levels. The activity was still found to be higher on day 5 as compared to day 1 (Figure 4.7).

Figure 4.3 PKC membranous activity in the distal and proximal colon of male Sprague Dawley rats killed 1 to 5 days for both injected and control groups. (n=3, mean ± SEM)

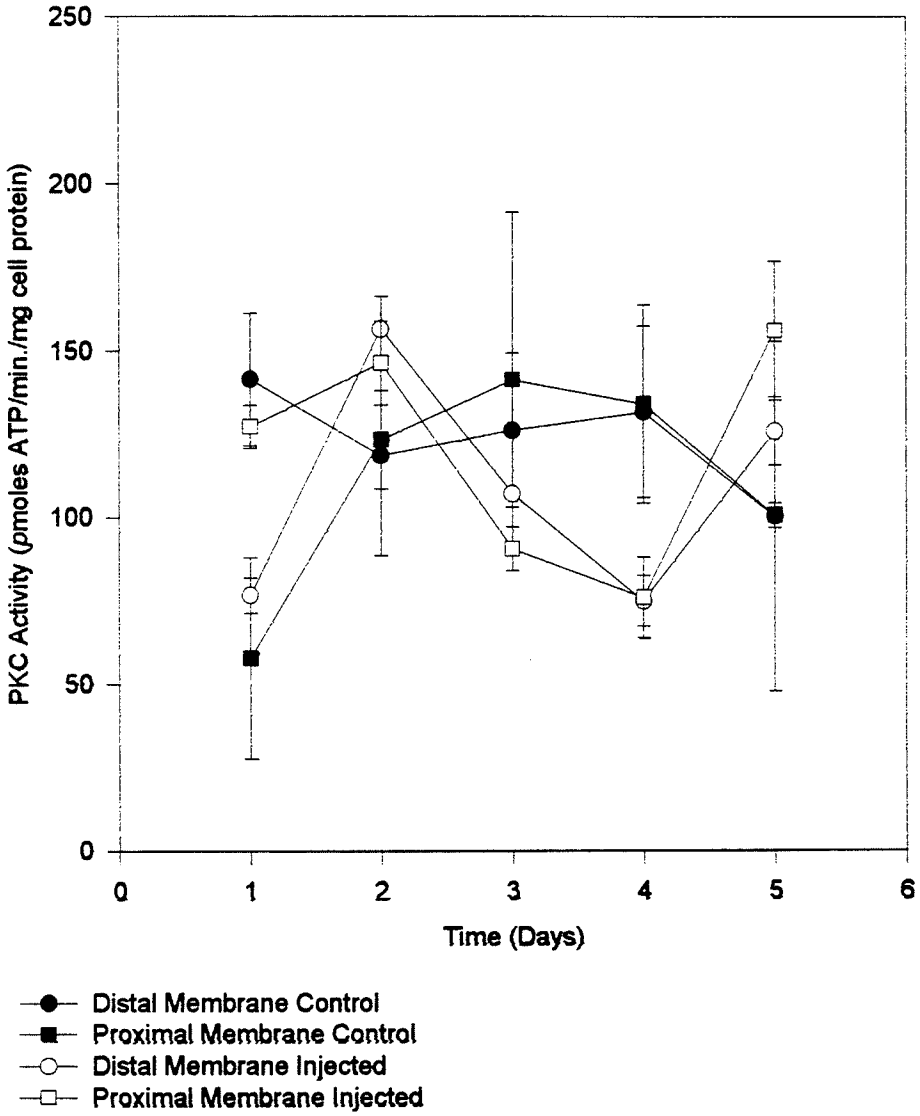


Figure 4.4 Combined distal and proximal PKC membranous activity in colons from male Sprague Dawley rats killed 1 to 5 days for both injected and control groups. (n=3, mean ± SEM)

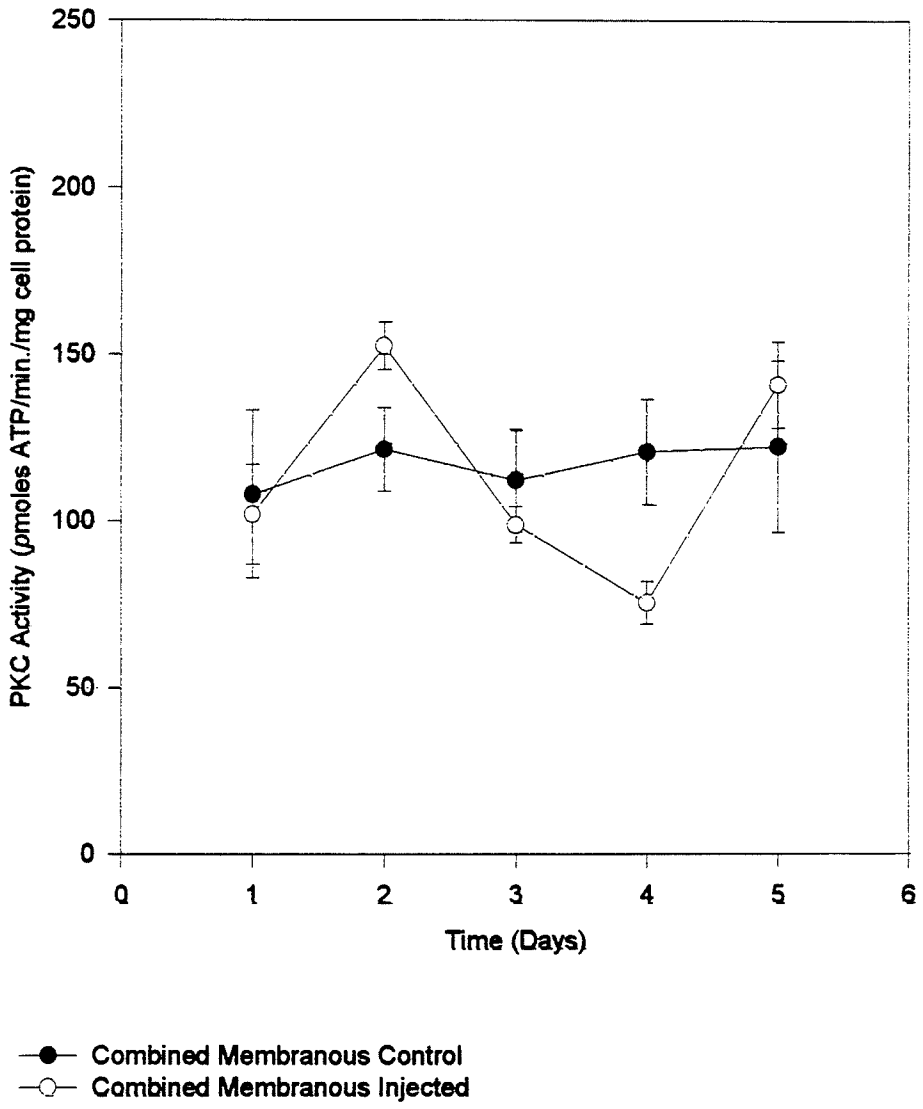


Figure 4.5 Overlapping bar graph of TK cytosolic activity in the distal and proximal colon of male Sprague Dawley rats killed 1 or 5 days for both injected and control groups. (n=3, mean ± SEM)

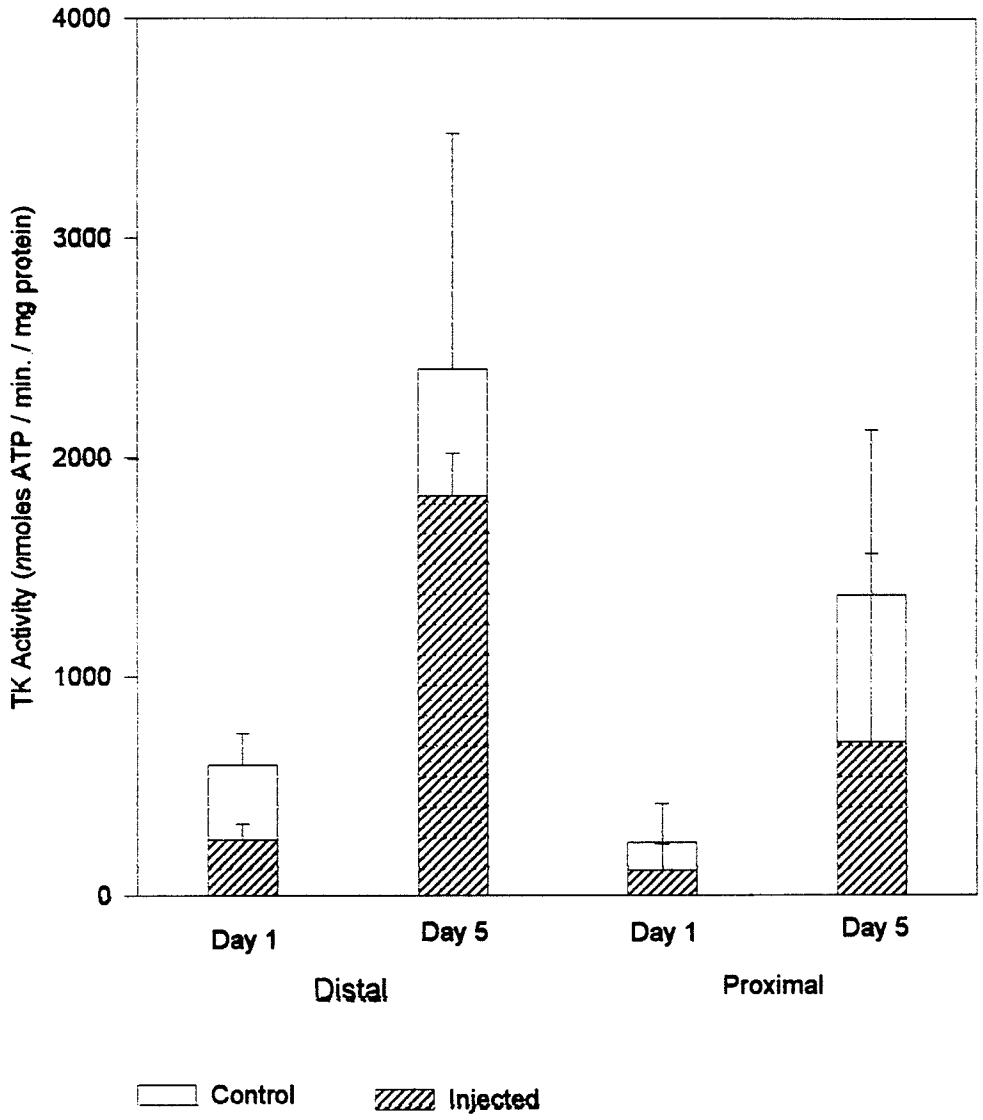


Figure 4.6 Overlapping bar graph of TK membranous activity in the distal and proximal colon of male Sprague Dawley rats killed 1 or 5 days for both injected and control groups. (n=3, mean ± SEM)

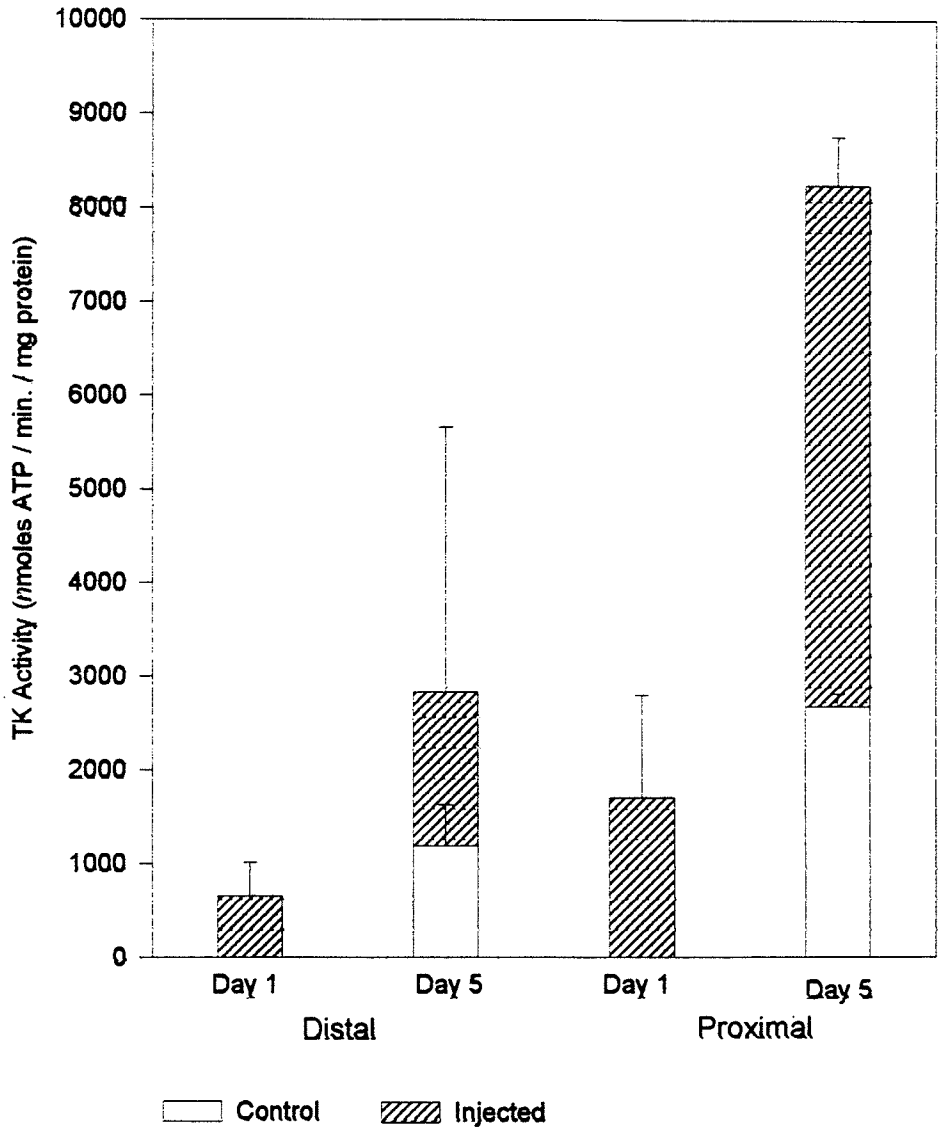
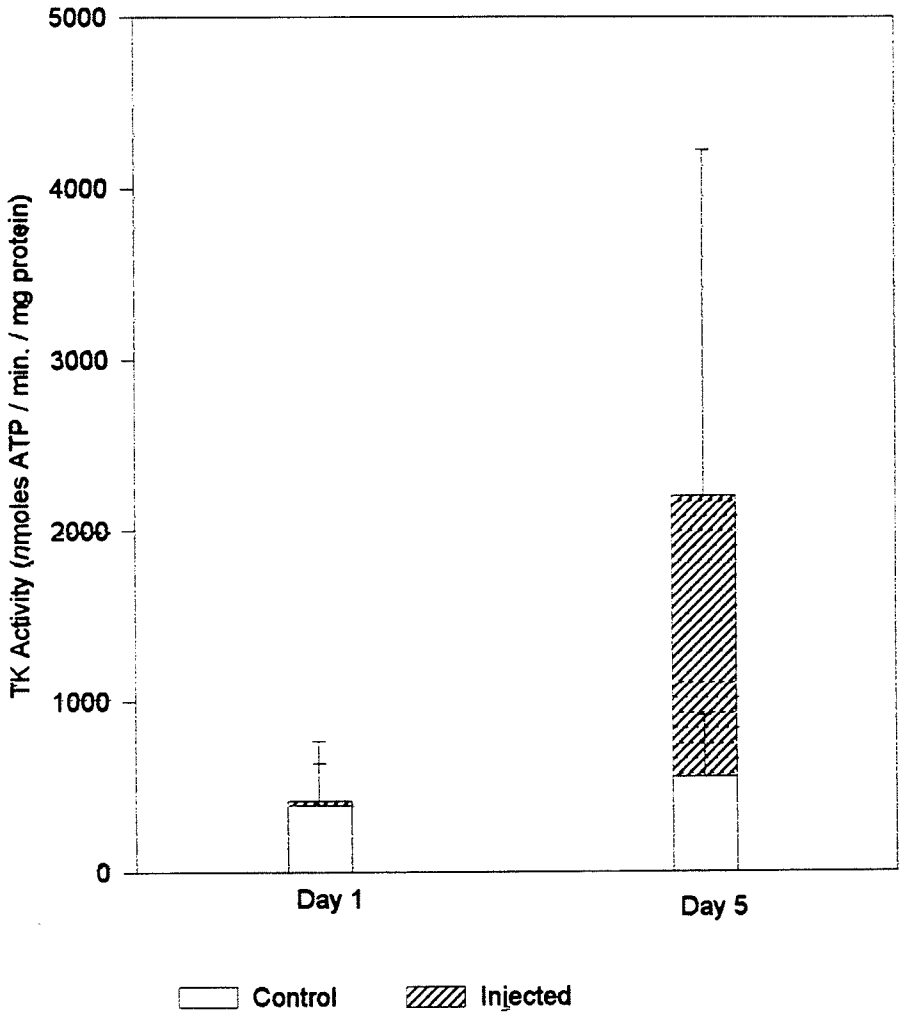


Figure 4.7 Overlapping bar graph of repeated TK membranous activity in the distal colon of male Sprague Dawley rats killed 1 or 5 days for both injected and control groups. (n=4 control, n=6 injected, mean ± SEM)



4.2.3 MAP Kinase Activity

MAP Kinase activity was assessed on immunoprecipitated membranous and cytosolic fractions using the MAP Kinase assay kit from Upstate Biotechnology (Lake Placid, NY). No significant differences were found in MAPK activity between the control and injected groups for the cytosolic fractions (Figure 4.8). The cytosolic activity was similar on all 5 days and no differences in regional activity were noted. Membranous MAP Kinase activity (Figure 4.9) was not significantly different between control and injected groups. The MAP Kinase membranous activity was similar on day 1, 2 and 3 at which point it increases for both control and injected groups on day 4 and 5. Distal and proximal regions showed similar trends in activity. Figure 4.10 demonstrates the relationship between membranous and cytosolic activity. Cytosolic and membranous activity are similar on day 1,2, and 3, but the activity is higher on day 4 and 5 for the membranous fractions.

4.3. Protein Expression

4.3.1 MAP Kinase Expression

MAPK isoforms were detected in both membranous (Figure 4.11) and cytosolic fractions. The Western blot for MAPK gave rise to two bands at approximately 42 and 44 kDa. The 44 kDa band appears to be more intense than the 42 kDa band in both the membranous and cytosolic fractions. No visible differences could be seen with respect

Figure 4.8 MAPK cytosolic activity in the distal and proximal colon of male Sprague Dawley rats killed 1 to 5 days for both injected and control groups. (n=3, mean ± SEM)

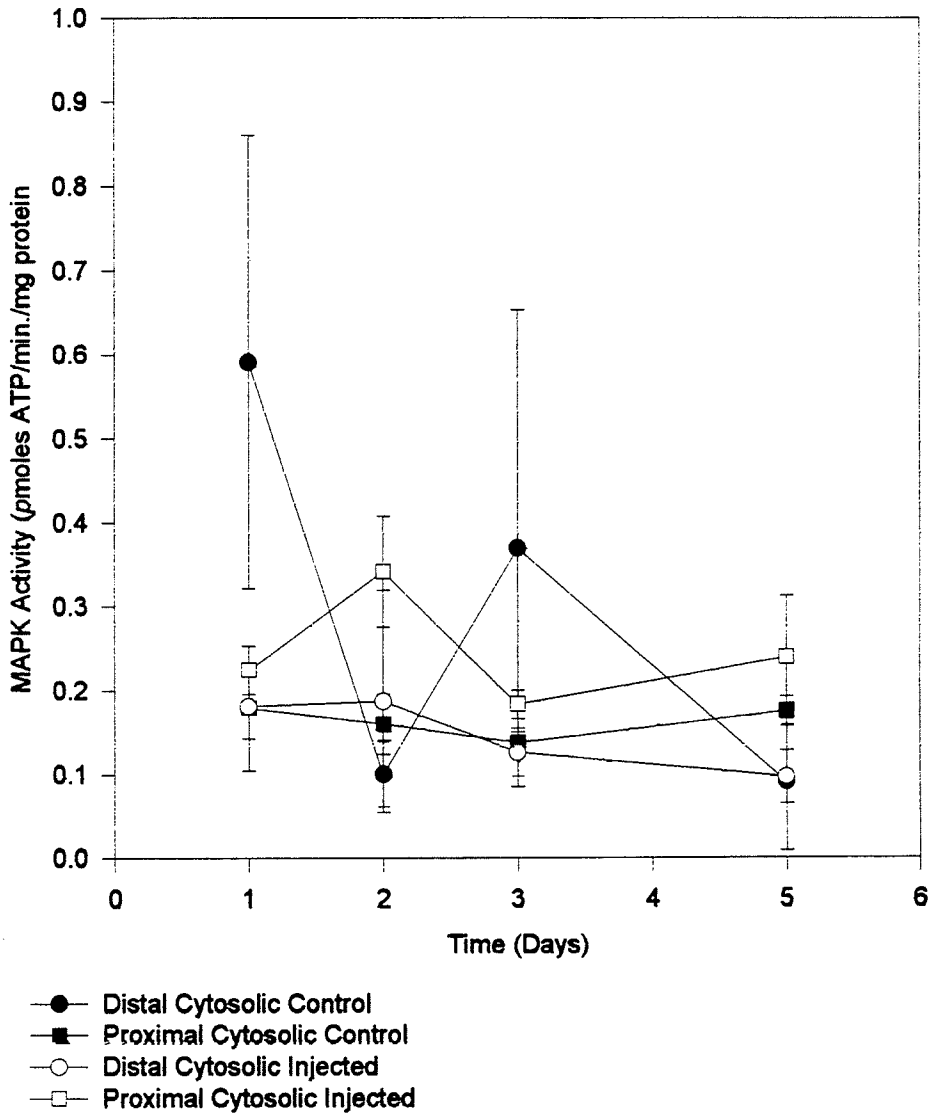


Figure 4.9 MAPK membranous activity in the distal and proximal colon of male Sprague Dawley rats killed 1 to 5 days for both injected and control groups. (n=3, mean ± SEM)

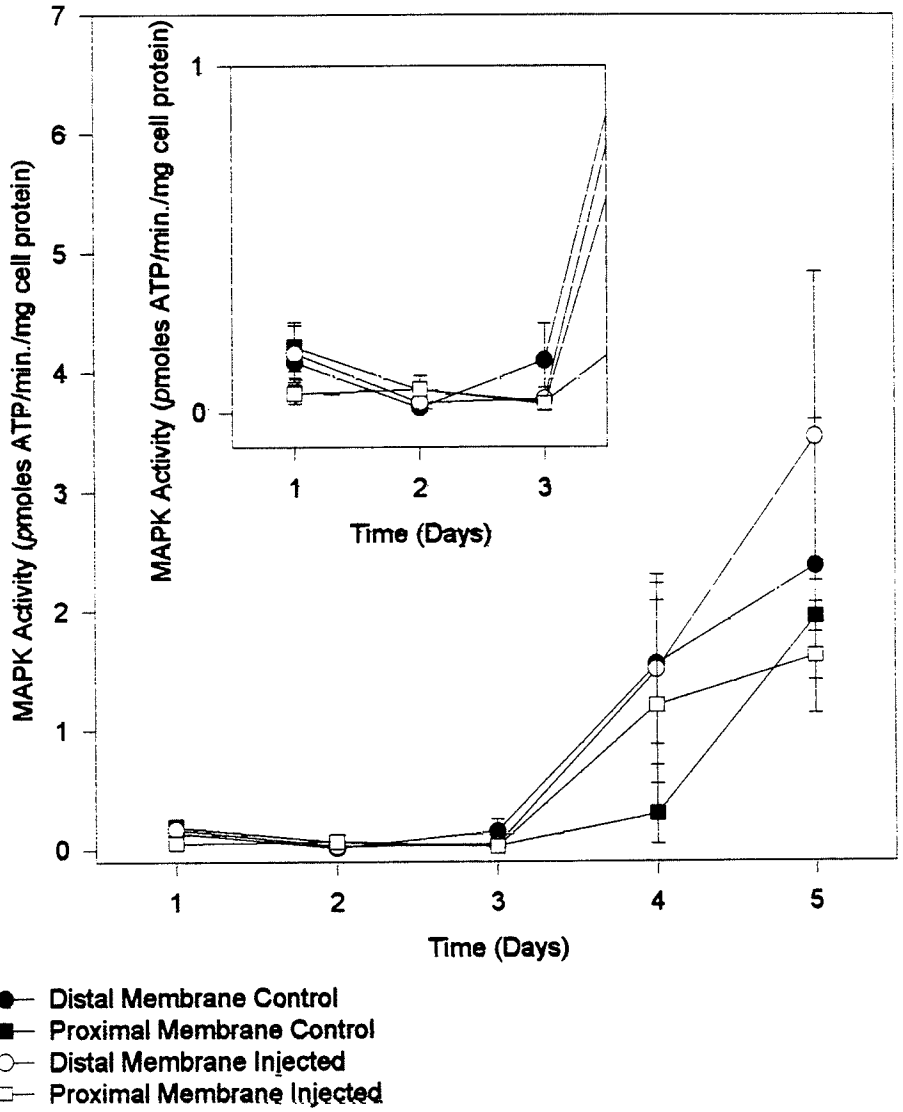


Figure 4.10 MAPK cytosolic and membranous activity in the distal colon of male Sprague Dawley rats killed 1 to 5 days for both injected and control groups. (n=3, mean ± SEM)

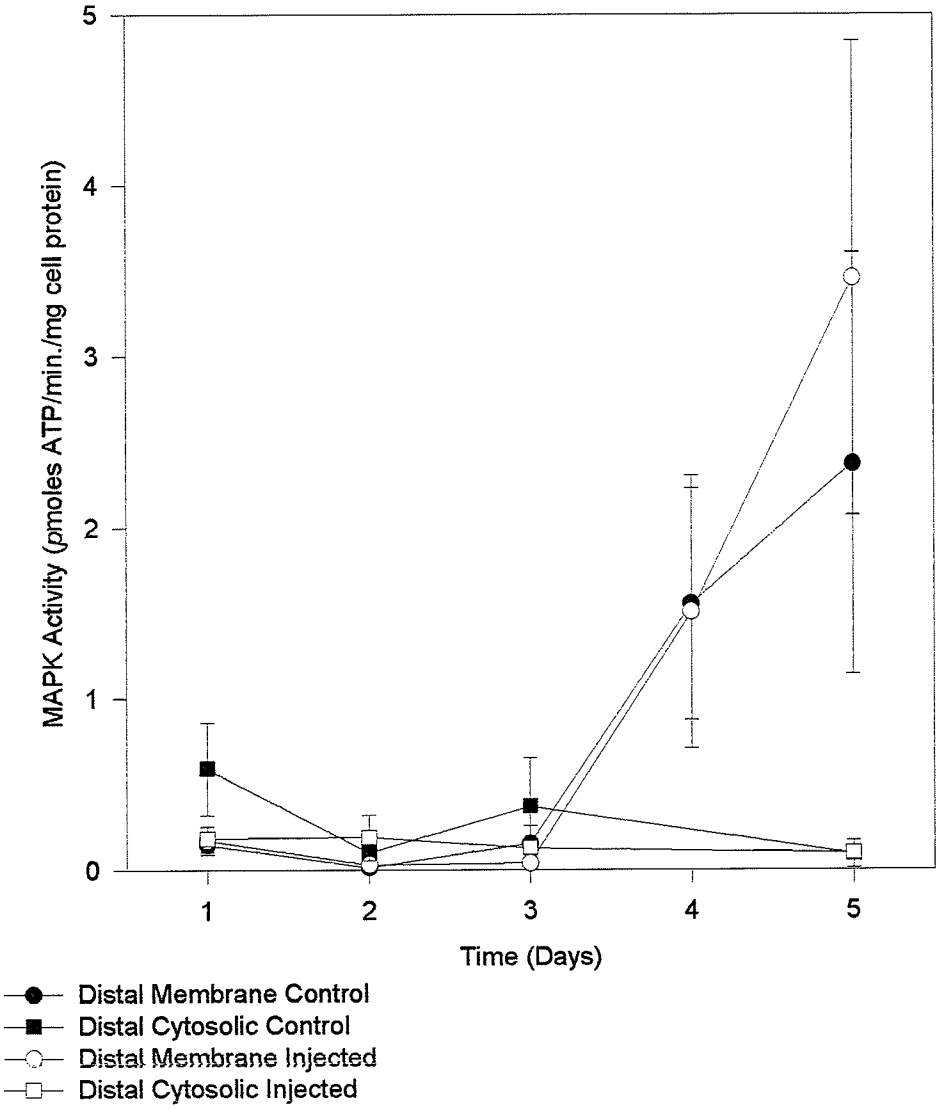
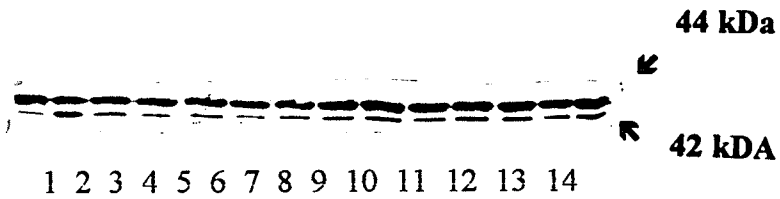


Figure 4.11 Representative Western blot depicting MAPK expression in the cytosolic fraction of the distal and proximal colon of male Sprague Dawley rats (n=3) killed 3 days after injection.



Legend:

Lane 1	Common sample to all gels
Lane 2	Positive control 3T3 cell lysate
Lane 3-8	Non-Injected controls
Lane 9-14	Injected samples

to expression of these isoforms in control and injected groups. Both isoforms were also detected in the positive control (3T3 cell lysate, Upstate Biotechnology, Lake Placid, NY) indicating that a sufficient amount of antibody was used.

The ratio of MAPK isoforms (42/44 kDa) expressed in the membranous and cytosolic fractions of colonic tissue from male Sprague Dawley rats killed 1 to 5 days after injection are summarized in Tables 4.1 and 4.2 respectively. The ratio of MAPK (42/44 kDa) isoforms is lowest on day 2 in the injected group for both membranous and cytosolic fractions. The ratio then increases to control levels by day 3.

4.3.2 Phosphotyrosine (P-tyr) expression

The general trend in the injected samples was that P-tyr expression either increased in the distal colon or decreased in the proximal colon or both compared to the control group. Of particular interest is the P-Tyr expression data for the 43kDa (cytosolic) and 44-47kDa (membranous) proteins as they likely represent MAPK. For the cytosolic fraction, there is no change in expression in the control samples on Day1, 3, or 5, and for the injected samples on Day 3 and 5 of the 43kDa protein. For the membranous fraction, the control and injected samples appear to change in similar ways on Day 1, 3 and 5 for the 44-47kDa proteins. For further details refer to Appendix Table 7.1 and 7.2.

Table 4.1 The ratio of MAP Kinase isoforms (42/44 kDa) expressed in the membranous fractions of the distal and proximal colon of male Sprague Dawley rats killed 1 to 5 days after injection.

Days	Control[1]		Injected[1]	
	Distal	Proximal	Distal	Proximal
Day 1	0.190 ± 0.057	0.300 ± 0.042	0.237 ± 0.065	0.295 ± 0.015
Day 2	0.183 ± 0.032	0.200 ± 0.057	0.100 ± 0.053	0.110 ± 0.061
Day 3	0.290 ± 0.020	0.303 ± 0.025	0.247 ± 0.025	0.223 ± 0.058
Day 4	0.295 ± 0.007	0.330 ± 0.070	0.323 ± 0.084	0.240 ± 0.070
Day 5	0.270 ± 0.010	0.223 ± 0.021	0.237 ± 0.023	0.217 ± 0.032

[1] Values are expressed as the mean ± SD. (n=3, per group)

Table 4.2 The ratio of MAP Kinase isoforms (42/44 kDa) expressed in the cytosolic fractions of the distal and proximal colon of male Sprague Dawley rats killed 1 to 5 days after injection.

Days	Control[1]		Injected[1]	
	Distal	Proximal	Distal	Proximal
Day 1	0.297 ± 0.049	0.293 ± 0.039	0.347 ± 0.068	0.307 ± 0.015
Day 2	0.223 ± 0.055	0.287 ± 0.132	0.197 ± 0.072	0.150 ± 0.050
Day 3	0.287 ± 0.021	0.310 ± 0.027	0.220 ± 0.040	0.273 ± 0.042
Day 4	0.280 ± 0.096	0.227 ± 0.083	0.233 ± 0.025	0.260 ± 0.026
Day 5	0.253 ± 0.115	0.220 ± 0.066	0.323 ± 0.015	0.243 ± 0.110

[1] Values are expressed as the mean ± SD. (n=3, per group)

4.4. Tumor Studies

4.4.1 Tumor Parameters

Tumor parameters were analysed at 29 weeks after injection and the results are summarized in Table 4.3. Also, a table summarizing the size and location of the tumor can be found in the Appendix (Table 7.3). The total number of tumors was found to be (significantly) higher in the high Ca group(2.0%) as compared to the normal Ca group (0.5%)(31 vs. 13). The colonic tumor incidence was not found to be significantly different between the two dietary groups (% animals with tumors). The high Ca group (2.0%) was found to have a significantly($p<0.01$) higher numbers of tumors in this range as compared to the normal Ca group (0.5%).

4.4.2 MAP Kinase Expression in Tumors

The MAP Kinase isoforms were expressed in control tissue and tumors from both dietary groups. Two bands were detected at approximately 42 and 44 kDa. The ratio of MAP Kinase isoforms (42/44 kDa) found in tumors from both high Ca (2.0%) and normal Ca (0.5%) groups are summarized in Table 4.4. The high Ca group (2.0%) was found to have a significantly higher ($p\leq0.01$) percentage of tumors with the ratio of 42/44 kDa MAP Kinase isoforms ≥0.79 as compared to the normal Ca group (0.5%)(73.0% vs. 16.7%). Expression of both the 42 kDa and the 44 kDa MAPK isoforms was increased in all small tumors found in the high calcium group. The 42 kDa MAPK isoform

Table 4.3 Colon tumor multiplicity in male Sprague Dawley rats fed a normal Ca (0.5%) or high Ca diet (2.0%) and killed 29 weeks after injection.[1] (n=9 per group)

Tumor Parameter	Diet Group	
	Normal Ca (0.5%)	High Ca (2.0%)
Total # of tumors	13	31
Tumor incidence	6 out of 9	8 out of 9
# of tumors per tumor bearing rat [2]	2.33 +/- 0.82	3.88 +/- 1.73*

[1] Dietary treatments were initiated 25 weeks after injection
[2] Values are expressed as mean +/- SD
* Denotes significant difference between high Ca and normal Ca group ($p<0.01$) using Chi square analysis for the frequency of tumors ≥ 2.

Table 4.4 The ratio of the expression of MAP Kinase isoforms 42/44 in colonic tumors from Sprague Dawley rats fed either a normal Ca (0.5%) or high Ca (2.0%) diet and killed 29 weeks after injection.[1]

Diet Group	Ratio (42/44 kDa)[2]
Normal Ca (0.5%) n=6	0.562+/-0.196 (0.26-0.79)
High Ca (2.0%) n=15	0.885+/-0.333 (0.26-1.36) *

[1] Dietary treatments were initiated 25 weeks after injection
[2] Values are expressed as mean +/- SD (range)
* Denotes significant difference between normal Ca and high Ca groups ($p<0.01$) using Chi square analysis for the frequency of tumors with a ratio of 42/44 MAP Kinase isoforms ≥ 0.79.

(9004 tumor vs 3805 control) had a greater increase in expression than the 44 kDa MAP isoform (7628 tumor vs 5244 control). Large tumors in the distal colon, regardless of diet, were also found to a higher ratio of MAPK expression (and both isoforms) than in the proximal colon. A table summarizing the absolute values of MAPK expression can be found in the Appendix Table 7.4

Chapter 5

Discussion

5. Discussion

This thesis was undertaken to examine the changes in mitogen activated protein kinases in colonic tissue and tumors after carcinogen exposure. MAPK's are thought to play an important role in colon carcinogenesis. The main findings of this research in relation to the specific objectives were: 1) cell proliferation and apoptosis were found to have an inverse relationship; 2) observed enzymatic parameters in normal and carcinogen treated colonic mucosa the first 5 days following carcinogen treatment revealed no significant differences in MAPK activity or expression or in PKC activity. However, TK activity in the carcinogen injected group was found to be significantly higher, approximately 3 times that of control, by day 5 in the proximal colonic tissue; 3) in the carcinogen treated group a high calcium diet introduced late in the study resulted in an increased tumor burden as compared to the normal calcium diet group. Further, an elevated level of MAPK expression was found in tumors in the high calcium diet group.

Cell proliferation is one of the most commonly used risk markers in the study of colon cancer. Carcinogen injection resulted in an initial decrease in proliferation, followed by a recovery or compensatory proliferation in the days following injection. Not surprisingly, the apoptosis results are inversely related to the proliferation data. Cell death peaked when cell proliferation was at its lowest, and as cell death returned to control levels, an increase in proliferation was observed. Other studies have observed similar trends in which carcinogen induced cell death was accompanied by a decrease in cell

proliferation in the first 24 hours following carcinogen injection (Bird, personal communication). The importance of studying these events was that the carcinogen did effect the proliferative area of the crypts, where the initiated cells appear. It is plausible that the enhanced cell proliferation evident on day 5, resulted in establishment of the mutational events.

It was important to assess how the kinases involved in cell signaling, responded to cell death and proliferation, therefore PKC, TK and MAPK were examined. PKC activity was measured only in the membranous fraction, as this fraction has been previously shown to contain the active form of PKC. PKC has received a great deal of attention for its' role in cell growth and proliferation and is generally associated with increased cell proliferation (Hug and Sarre, 1993; Sando *et al.*, 1992). The results observed here showed that the PKC activity was highest when cell proliferation was at its' lowest. Other studies have seen similar results and do not support the contention that increased PKC activity is associated with increased cell proliferation (Lasko, 1997, Craven & DeRubertis, 1992).

Tyrosine kinases have been implicated as playing an active role in cancer development. In relation to the cell proliferation data, membranous TK activity was low on DAY 1 when cell proliferation was at it's lowest, and high on DAY 5 when cell proliferation was at its' peak. The results here show that membranous TK activity is elevated 5 days post carcinogen injection, and that little or no change in activity occurred in the cytosolic fraction during this time frame. It is important to note that the observed

TK activity was opposite to that of PKC activity. In addition proximal colonic regions responded differently as opposed to the distal colonic regions. Specifically the membranous TK activity was higher in the proximal colon on day 5 than in the distal. The opposite trend was observed in the cytosolic fraction. This emphasizes the fact that proximal colonic regions differ from distal colonic regions. This trend is also reflected in tumor incidence and distribution data from the second study. Elevated TK activity has been seen in numerous studies including those involving the colonic mucosa of carcinogen injected rats (Rao *et al.*, 1993: Singh *et al.*, 1992; Arlow *et al.*, 1989), and human colonic carcinomas (Sankanoue *et al.*, 1990). Few studies have looked at cytosolic and membranous activity separately, making it more difficult to interpret these results. Several non-SRC (membranous) tyrosine kinases such as the EGF receptor, Insulin-like growth factor receptor and Insulin receptor have been found to have elevated intrinsic TK activity, supporting the finding of increased membranous TK activity, and suggesting they are important in colon cancer development (Radinsky *et al.*, 1995). SRC, tyrosine kinases which are located in the cytosol, have been reported to be elevated in the epithelium of patients with ulcerative colitis (Cartwright *et al.*, 1990) suggesting that they may be involved in the early stages of cancer development. With this in mind, one would have expected to see an increase in the cytosolic TK activity. Perhaps, cytosolic TK activity takes longer to react than the membranous TK activity, and the 5 day time frame was not a sufficient length of time to observe any significant changes. Therefore, a study which

looks at TK activity over a extended duration may provide more insight into whether or not cytosolic TK activity is involved in the early stages of the carcinogenic process.

MAPK's are an integral part of the Ras-mediated signal transduction pathway, and are involved in the regulation of cell proliferation and differentiation (Crews and Erickson, 1993). The results here show that there is no change in MAPK activity in the cytosolic fraction on DAY 1 through to DAY 5, and that there was no change in MAPK activity in the membranous fraction on DAY 1 through DAY 3, but the activity increased on DAY 4 and 5 for both the control and injected samples. This study is the first study which looked at MAPK activity in the early stages of colon carcinogenesis, and therefore there is no data to which the results can be compared. The increase in the membranous MAPK activity in the injected sample was unexpected. If MAPK was to play a role in cell proliferation one would expect to see an increase in the cytosolic activity, as this is where it is activated, and where several of it's cellular targets are. In relation to the cell proliferation data, membranous MAPK activity is low on DAY 1 when cell proliferation is at it's lowest, and higher on DAY 5 when cell proliferation is at its' peak. The results here should be here should be taken with caution due to the limited number of samples used and the sensitivity of the method to the experimental conditions, both of which might explain the variability in the results. Further research is required to determine the role of MAPK in the early stages of the colon carcinogenic process.

The results for MAPK expression were similar for both the membranous and

cytosolic fractions, and no regional differences in expression were observed. There have been no other studies to date which have looked at MAPK expression in the early stages of colon carcinogenesis. In terms of the cell proliferation data, MAPK expression was lowest on DAY 2 when cell proliferation was lowest, and returned back to control levels on DAY 3 when the cells were gearing up for proliferation. MAPK activity corresponded well with the expression data for the membranous fraction, however no relation was seen between the cytosolic activity and MAPK expression data.

Quantification of P-42 and P-44 kDa MAPK isoforms revealed that there exists numerous phosphorylated proteins within the colonic tissues. The results of the P-Tyr expression data indicate that some of the P-Tyr proteins are normally expressed to different degrees in the distal and proximal colon, and that the general trend in the injected rodents was that P-Tyr expression either increased in the distal colon or decreased in the proximal colon (or both), with respect to controls. These regional differences in P-Tyr expression may account for some of the differences seen in tumor outcome in the distal and proximal colon. These results suggest that these two regions behave like two distinct organs.

Of particular interest from this P-Tyr expression data are the 43 kDa (cytosolic) and 44-47 kDa (membranous) proteins, as they likely represent MAPK. As indicated previously MAPK is activated by phosphorylation on both tyrosine and threonine residues (Davis, 1993). In the cytosolic fraction, there is no change in expression in the control

sample on Day 1, 3, or 5 for the 43 kDa protein, which is what is seen for the cytosolic MAPK activity. For the injected samples, with exception of Day 1, no changes in P-Tyr expression are seen on Day 3 and Day 5 which again is what is seen for MAPK cytosolic activity. In terms of the membranous fraction, the control and injected samples both appear to change in similar ways over the 5 days for the 44-47 kDa proteins as is seen for the membranous MAPK activity.

The possible role of calcium as a chemopreventive agent has recently come into question. However, the results here show that calcium supplementation (2%) given at 25 weeks post carcinogen injection and feed for 4 weeks results in a significantly greater (31 vs. 13) number of tumors than in the control group. Similar results were seen in a study by Pence and colleagues in which calcium was introduced 38 weeks into the study and fed for the remainder (Pence *et al.*, 1995). As in this research, TI was not significantly altered, but tumor burden was increased compared to the controls.

A greater number of tumors were found in the proximal colon compared to the distal colon, regardless of the diet. This was expected, and may be the result of the proximal colon of Sprague Dawley rats being more sensitive to the carcinogen induced cancer model. This has also been seen in other studies which have used this model (Bird *et al.*, 1996, Lasko, 1997).

To date, no other studies have looked at the expression of MAPK in colonic tumors, in the presence of a high calcium diet. Results obtained indicate that calcium did

indeed have a positive effect on MAPK expression, in that there was an observed increase relative to controls. The ratio of P-42/P-44 isozyme expression was found to be significantly ($p \leq 0.01$) higher in the high calcium group for tumors with a ratio ≥ 0.79 as compared to the normal calcium group. The results indicate that all small tumors induced by increased calcium had an elevated ratio of P-42/P-44. Further, there was also an overall increase in expression of both isoforms. Large tumors found in the distal colon were also observed to have a higher ratio of MAPK isoforms than large tumors found in the proximal colon regardless of diet. However, only a small number of large tumors were found in each group, and further analysis is required to for more concrete statements to be made.

Due to the high number of smaller tumors in the high calcium group, the results suggest that high dietary calcium modulates kinase expression by increasing the expression of P-42 kDa MAPK isoform relative to P-44 kDa MAPK and by increasing the expression of both isoforms. An alternative interpretation of the results would be that a high calcium diet preferentially stimulates the growth of these lesions already characterized by elevated MAPK expression. These results indicate that MAPK may play a critical role in colon carcinogenesis. Further research is required to ascertain the involvement of MAPK and calcium in the carcinogenic process.

In conclusion this study has demonstrated that early events as measured by cell proliferation, cell death and kinase activity were transient in nature and failed to elucidate

any additional insights into their respective roles in colon carcinogenesis. PKC and TK activity were determined to oppose to one other. Proximal and distal colonic tissues differ in their response to carcinogen treatment. The long term study provided evidence that there are a group of lesions that responded in a positive (more lesions) manner to a high calcium diet. These tumors were found to have elevated levels of MAPK expression. Further research is required to elucidate whether or not the observed growth stimulation of the preneoplastic lesions was due to a direct effect of increased intracellular calcium on MAPK.

Chapter 6

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6.1 Literature Cited

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Appendix

7. Appendix

7.1 Vitamin mix (AIN-93-VX)

Vitamin	g/kg mix
Nicotinic acid	3.000
Calcium pantothenate	1.600
Pyridoxine-HCl	0.700
Thiamin-HCl	0.600
Riboflavin	0.600
Folic acid	0.200
D-Biotin	0.020
Vitamin B-12	2.500
Dry Vitamin E acetate	15.000
Dry Vitamin A palmitate	0.800
Vitamin D_3	0.250
Vitamin K	0.075
Powdered sucrose	974.655

7.2 Mineral Mix (AIN-93M-MX)

Mineral	mg/kg diet
Calcium	5000.0
Phosphorus	1992.0
Potassium	3600.0
Sulfur	300.0
Sodium	1019.0
Chloride	1571.0
Magnesium	507.0
Iron	35.0
Zinc	30.0
Maganese	10.0
Copper	6.0
Iodine	0.2
Molybdenum	0.15
Selenium	0.15

7.3. Buffers Used for the Separation of Membranous and Cytosolic Fractions

7.3.1 Cytosolic Buffer

25 mM Tris (pH 7.5)

5 mM EDTA

5 mM EGTA

0.25 M sucrose

15 mM mercaptoethanol

Protein inhibitors were added just before use at the following levels; leupeptin 0.5 *u*g/ml, aprotinin 2.0 *u*g/ml, trypsin 10.0 *u*g/ml. PMSF was added at a concentration of 0.25 mM.

7.3.2 Membranous Buffer

Cytosolic buffer with the addition of TritonX-100 at a level of 0.533%.

7.3.3 Buffer Used for Whole Cell Lysate - RIPA Buffer

50 mM Tris-HCl (pH 7.4)	150 mM NaCl
1.0% NP-40	1 mM EDTA
0.25% Sodium deoxycholate	1 mM NaF

Protein inhibitors were added just before use at the following levels ; leupeptin, aprotinin, and trypsin at 1 *u*g/ml. PMSF and Na_3VO_4 were added at a concentration of 1 mM.

7.4 Westerns

All reagents and recipes are from Bio-Rad.

7.4.1 30% Acrylamide/bis Mix

87.6 ml acrylamide (29.2 g/100 ml)

2.4 ml N'N-bis-methylene-acrylamide (0.8 g/100 ml)

Made up to volume with dH_2O and stored at 4°C in the dark.

7.4.2 10% Ammonium Persulfate (APS)

100 mg ammonium persulfate in 1ml dH_2O

7.4.3 10% Sodium Dodecyl sulfate (SDS)

10 g of SDS dissolved in and brought up to 100 ml with dH_2O, and stored at room temperature

7.4.4 Sample Buffer (8 ml)

dH_2O	3.8 ml
0.5 M Tris HCl (pH 6.8)	1.0 ml
glycerol	0.80 ml
10% SDS	1.6 ml
2-mercaptoethanol	0.40 ml
1% (w/v) bromphenol blue	0.40 ml

7.4.5 5% Stacking Gel

H_2O	3.4 ml
30% acrylamide mix	0.83 ml
0.5 M Tris Hcl (pH 6.8)	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

7.4.6 10% Separating Gel (10ml)

dH_2O	3.3 ml
30% acrylamide mix	4.0 ml
1.5 M Tris Hcl (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10%APS	0.1 ml
TEMED	0.004 ml

7.4.7 5X Running Buffer

Tris base	9.0 g
glycine	43.2 g
SDS	3.0 g

The above components were mixed and the volume was brought up to 600 ml with dH_2O. The solution was stored at room temperature and was diluted to 1x before use.

7.4.8 Transfer Buffer

Tris base	3.03 g
glycine	14.4 g
methanol	200 ml

The above components were mixed and the volume was brought up to 1000 ml with dH_2O. The solution was stored at 4°C.

7.4.9 Coomassie Blue Stain

0.1% Coomassie blue R-250

40% methanol

10% acetic acid

50% dH_2O

Gels were stained for atleast 1/2 hour.

7.4.10 Destaining Solution

40% methanol

10% acetic acid

50% dH_2O

Gel was destained in several changes of this solution over a few hours.

7.4.11 TBS-T Buffer

100 mM Tris HCl

0.9% NaCl

0.1% Tween 20

Solution was stored at room temperature.

7.4.12 India Ink Staining

Membranes were washed with TBS-T (3x30min) at 37°C and (2x30min) at room temperature. The membranes were then incubated overnight in a 0.1% India Ink stain in TBS-T. The membranes were then destained for 1/2 hour to 1 hour in TBS-T at room temperature.

7.5 MAPK Immunoprecipitation and Assay Buffers

7.5.1 Tris Buffer

50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4,

0.1% 2-mercaptoethanol, 1% TritonX-100, 50 mM sodium fluoride,

5 mM sodium pyrophosphate, 10 mM sodium β-glycerol phosphate, 0.1 mM PMSF

Aprotinin, trypsin, and leupeptin were all added at a level of 1 *u*g/ml.

7.5.2 Assay Dilution Buffer (ADB)

20 mM MOPS (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol

7.6 Phosphotyrosine (P-Tyr) Expression

The results for P-Tyr expression in the distal and proximal colons of male Sprague Dawley rats killed 1, 3, and 5 days after injection are summarized in Table 7.1 (cytosolic fractions) and Table 7.2 (membranous fractions). No attempt to quantify the bands was made, and the results summarized are strictly observational. Only bands which were visibly distinct are described. It is interesting to note that some of these proteins are normally expressed to different degrees in the distal and proximal colon.

In the cytosolic fractions, most changes between the control and injected samples occurred on day 1. The general trend was that P-Tyr expression in the injected samples either increased in the distal colon or decreased in the proximal colon (or both), with respect to their corresponding controls. The exception would be the 32 kDa protein in which P-Tyr expression on day 1 appears to increase in both the distal and proximal colon in the injected samples. However, like the control on this day, P-Tyr expression remained more intense in the distal colon. On day 3, P-Tyr expression for this 32 kDa protein is more intense in the proximal colon, and by day 5 it is approximately equal in both the distal and proximal colon. The only other change noted for the cytosolic fractions was that on day 5 P-Tyr expression changed in both the control and injected samples for the 23 and 24 kDa proteins.

For the membranous fractions the only changes which occurred on day 1 were for P-Tyr expression in the 59, 31 and 34 kDa proteins. The 59 kDa protein, like the control,

Table 7.1 Phospho-tyrosine (P-tyr) expression in the cytosolic fractions of the distal and proximal colon of male Sprague Dawley rats killed 1, 3, and 5 days after injection. (n=3)

~M.Wt.	DAY 1	DAY 3	DAY 5
70-71 kDa (doublet)	Control - present Injected - present, more intense in distal colon	Control - present Injected - present	Control - present Injected - present
58 kDa	Control - present, more intense in proximal colon Injected - present	Control - present, more intense in proximal colon Injected - present, more intense in proximal	Control - present, more intense in proximal colon Injected - present, much more intense in prox.
46 kDa	Control - present Injected - present, more intense in distal colon	Control - present Injected - present	Control - present Injected - present
43 kDa	Control - present, more intense in proximal colon Injected - present, more intense in distal colon	Control - present Injected - present	Control - present Injected - present
32 kDa	Control - present, more intense in distal colon Injected - present, more intense in distal colon, and than control	Control - present, more intense in distal colon Injected - present, much more intense in proximal colon	Control - present, more intense in distal colon Injected - present
23-24 kDa (doublet)	Control - present, much more intense in prox. Injected- present, more intense in proximal colon ,less intense than control	Control - present, much more intense in proximal colon Injected - present, much more intense in proximal	Control - present, a bit more intense in distal colon Injected - present, a bit more intense in distal colon

Table 7.2 Phospho-tyrosine (P-tyr) expression in the membranous fractions of the distal and proximal colon of male Sprague Dawley rats killed 1, 3, and 5 days after injection. (n=3)

~M.Wt.	DAY 1	DAY 3	DAY 5
71 kDa	Control - present Injected - present	Control - present Injected - present, less intense	Control - present Injected - present
59 kDa	Control - present Injected - present, less intense	Control - present Injected - present, more intense in distal colon	Control - present Injected - present
52 kDa	Control - present Injected - present	Control - present Injected - present	Control - present Injected - present, more intense in distal and than in the control
44-47 kDa (doublet)	Control - top band equal in all samples, bottom band more intense in proximal colon Injected -same as control	Control - both bands present in equal amounts Injected - top band more intense in distal colon, bottom band equal in all samples	Control - both bands equal in all samples, bottom band more intense Injected - same as in the control, but more intense
31-34 kDa (doublet)	Control - present in all samples, both bands more intense in proximal colon Injected - present in all samples in equal amounts	Control - present in all samples, both bands more intense in proximal colon Injected - present in all samples in equal amounts, less intense than in control	Control - present in all samples, bottom band more intense Injected - same as in the control, but more intense
26-29 kDa (doublet)	Control - present in all samples, both bands more intense in proximal colon Injected - same as in the control	Control - present in all samples, both bands more intense in proximal colon Injected - same as in the control, less intense	Control present in all samples, both bands more intense in the proximal colon Injected - same as in the control

was present in all samples (distal and proximal) in equal amounts, but the expression was less intense in the injected samples. P-Tyr expression in the 31 and 34 kDa proteins was found to be more highly expressed in the proximal colon for the control samples where as the expression for the injected samples was equally distributed. Thus, it appears that either P-tyr expression was increased in the distal colon or decreased in the proximal colon for these 31 and 34 kDa proteins. On day 3 changes were observed in all the proteins except the 44 and 52 kDa proteins. The 71, 26 and 29 kDa proteins were expressed in the same proportions as their respective controls but were less intense in the injected samples. The 59 and 47 kDa proteins which are equally expressed in their control samples were found to be more highly expressed in the distal colon of the injected samples. Expression of the 31 and 34 kDa proteins was also altered. In the control samples this protein is more highly expressed in the proximal colon, and on day 3 was found to be equally distributed in the injected samples. Expression of these proteins was also at a lower intensity than in the control samples. On day 5 differences between control and injected samples were seen for the 52, 47, 44, 34 and 31 kDa proteins. The P-Tyr expression for the 31, 34, 44 and 47 kDa proteins was more intense than their respective controls, but followed the same distribution patterns. Expression of the 52 kDa protein in the injected sample was also more intense than its respective control, but the distribution of this protein was altered. In the control samples the 52 kDa protein is equally distributed, however in the injected samples there is increased expression in the distal fractions. It should be noted that the

expression of P-Tyr in the control samples for the 44 and 47 kDa proteins changes from day to day and for the 31 and 34 kDa proteins is different on day 5.

7.7 Longterm Study Tumor Distribution Data and MAPK Expression in Tumors

Tumor distribution data and sizes are summarized in Table 7.3 and 7.4 below. MAPK expression of the 42 and 44 kDa isoforms for tumor fed a normal (0.5%) or high (2.0%) Ca dieat are summarized in Table 7.5

Table 7.3 Tumor distribution data and size for tumors from Sprague Dawley rats fed a normal Ca (0.5%) diet and killed 29 weeks after injection.

Location / Size	Large (> 5mm x 4 mm)	Small (< 3mm x 3 mm)	Total
First 5 cm	1	6	7
6 cm+	5	1	6
Total	6	7	13

Table 7.4 Tumor distribution data and size for tumors from Sprague Dawley rats fed a high Ca (2.0%) diet and killed 29 weeks after injection.

Location / Size	Large (> 5mm x 4 mm)	Small (< 3 mm x 3 mm)	Total
First 5 cm	4	8	12
6 cm +	12	7	19
Total	16	15	31

Table 7.5 Absolute area values of the expression of the 42 and 44 kDa MAPK isoforms in colonic tumors from Sprague Dawley rats fed either a normal calcium (0.5%) or high calcium (2%) diet and killed 29 weeks after injection. The non-injected control group was fed a normal calcium (0.5%) diet.

	Proximal Area of band 42kDa, 44kDa	Distal Area of band 42kDa, 44kDa
Normal Diet	4218, 2449	12367, 8200
	3581, 1786	6484, 5153
	4490, 2172	
	7964, 5166	
High Calcium Diet	4868, 1891	9722, 8626
	4969, 2297	10430, 8707
	3841, 992	10105, 10607
	7327, 9701	7876, 8197
	7121, 9706	8282, 9581
	4218, 2525	11220, 10412
	11656, 9301	
	11196, 11337	
Control	6399, 3485	
	5244, 3805	
	7782, 5683	