INVESTIGATION OF THE MULTISTEP PROCESS OF COLON

CARCINOGENESIS AS AFFECTED BY DIETARY FAT AND ENERGY

BY

CATHERINE MICHELLE LASKO

A Thesis

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Catherine Michelle Lasko   1997 (c)

ABSTRACT

The main objective of this dissertation was to explore the role of dietary fat and energy restriction (ER) on the multistep process of colon carcinogenesis and to investigate the cellular and molecular features of the morphological determinants of various preneoplastic and neoplastic states. It was hypothesized that ER and fat would exert different effects on the growth of preneoplastic lesions depending on their phenotypic features and that their growth modulating abilities would be mediated via alteration of biochemical events known to be involved in cell growth and differentiation. A series of studies was conducted in male F344 rats to investigate the effects of ER (20%) in low (LF) and high fat (HF) corn oil diets on the number and growth characteristics of aberrant crypt foci (ACF), microadenomas and adenocarcinomas representing early, intermediate, and late stages of colon carcinogenesis respectively. Changes in dietary fat during the early stages exerted measurable responses on ACF development more rapidly than ER. Feeding a low fat diet during the early time point soon after initiation of colon carcinogenesis permitted the modulation of ACF, microadenomas and tumors by changes in fat and energy during the intermediate stages of carcinogenesis. Upon exposure of the animals to high fat diets during the early time point, a more stringent and growth conducive environment for modulation of ACF, it was demonstrated that ER in conjunction with LF retarded the appearance of macroscopic lesions. The enzymes protein kinase C and tyrosine kinases did not exhibit specific or consistent changes that were attributable to ER or fat associated growth responses. Employing the techniques of reverse

transcription polymerase chain reaction, biological difference were demonstrated
among the morphological determinants of the multistep process of colon
carcinogenesis. The findings of the present research demonstrated that biological
responses elicited by ER and fat are separable and that the amenability of the disease
process to a growth modulatory environment depends on its biological state.

ACKNOWLEDGEMENTS

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PUBLISHED MATERIAL

Lasko, C.M. and Bird, R.P. (1995) Modulation of aberrant crypt foci by dietary fat and caloric restriction: the effects of delayed intervention. Cancer Epidemiol. Biomark. Prev. 4:49-55.

Bird, R.P., Yao, K., Lasko, C.M. and Good, C.K. (1996) Inabiiity of low-or high-fat diet to modulate late stages of colon carcinogenesis in Sprague-Dawley rats. Cancer Res. 56:2896-2899.

LIST OF ABBREVIATIONS

ACF	aberrant crypt foci
ANOVA	analysis of variance
AOM	azoxymethane
ATP	adenosine triphosphate
DAG	diacylglycerol
DEPC	diethylpyrocarbonate
ECL	enhanced chemiluminescence
EGFR	epidermal growth factor
EGFR-TK	epidermal growth factor associated tyrosine kinase
HF	high fat
HFER	high fat energy restricted
LF	low fat
LFER	low fat energy restricted
LI	labelling index
MA	microadenoma
MI	mitotic index
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PKC	protein kinase C
RT-PCR	reverse transcriptase polymerase chain reaction
s.c.	sub cutaneous
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel
SEM	standard error of the mean
TA	tumors per animal
TBA	tumors per tumor bearing animal
TCA	trichloroacetic acid
TK	tyrosine kinase

1. INTRODUCTION

Cancer of the colon is the second leading cause of cancer deaths in North America (Schatzkin et al., 1996). Once the individual is identified to have large bowel cancer, it is estimated that fewer than 60% will survive past 5 years (Fernandez et al., 1996). It has been hypothesized that up to 90% of colon cancer could be prevented by dietary modification (Doll and Peto, 1981). Therefore, prevention of the disease may be the best route taken to circumvent the development of colon cancer. Incidentally, diet has become a paramount issue for several recent epidemiological studies (Lanza et al., 1996; Fernandez et al., 1996; Schatzkin, et al., 1996) measuring the occurrence and re-occurrence of colonic polyps.

With the advent of superior techniques in molecular biology, the genetic basis for colon cancer has become established (Fearon and Jones, 1992; Fearon, 1994; Vogelstein et al., 1988, 1989). However, there is a lack of knowledge pertaining to the potential effects of diet on the expression of genetic aberrations in colon cancer. The effects of diet on colonic tumor incidence has been investigated in several studies (Bird et al., 1996; Kumar et al., 1991; Reddy et al., 1987) employing animal models and have identified potential modulators of the disease. The mechanisms by which diet alters the disease outcome (tumor development) are not well elucidated and has been suggested to be the consequence of a lack of a method to study the step-wise development of colon cancer (Bruce, 1987; Harris, 1991).

In 1987, Bird (1987) initially postulated that aberrant crypt foci (ACF) were preneoplastic colonic lesions that exhibited the potential, as a bioassay, to study the

stepwise development of colon cancer and identify potential modulators of the disease, including diet. Over the past decade, several studies have supported that contention that ACF are precursor lesions of colon cancer (McLellan and Bird, 1988a, 1988b; McLellan er al., 1991a, 19991b; Pretlow et al., 1991, 1992a, 1992a, 1992b;). Incidentally, the effect of various modulators of colon cancer on ACF growth characteristics has not been consistent and the value of ACF as precursor lesions has come into question (Carter et al., 1994; Hardman et al., 1991). However, a few studies have provided evidence suggesting that experimental protocol may significantly affect the pattern of ACF growth and development, and their ability to predict tumor incidence (Bird, 1995b; Magnuson and Bird, 1993; Magnuson et al., 1993). ACF have been demonstrate to exhibit biological heterogeneity with certain populations more amenable to enhancement or inhibition by modulators of the disease (Bird, 1996; Magnuson et al., 1993). Such observations strengthen the need for future studies exploring the role of ACF in colon carcinogenesis.

Restriction of dietary energy has long been associated with decreased end tumor incidence in several tissues (Tannenbaum, 1945a, 1945b; Tannenbaum et al., 1953). Recent investigations have substantiated the ability of energy restriction (ER) to significantly reduce chemically induced rat colon tumor incidence in diets with varying fat content (Klurfeld et al., 1987; Kumar et al., 1990; Kritchevsky et al., 1986; Pollard and Luckert, 1985; Reddy et al., 1987). ER has also been implicated in the inhibition of age associated changes in cellular functions resulting in decreased susceptibility for tumor development (Weindruch et al., 1991). However, the specific mechanism(s) by

which ER precludes tumor incidence have not been elucidated. In addition, the effects of ER in the preneoplastic rat colon have not been investigated.

This dissertation evolved from the existing data supporting ER as a colonic tumor inhibitor and the lack of data exploring the effects of ER on the preneoplastic colon. Employing the ACF system to study the effects of ER in diets of varying fat content provides a means by which the varying stages of colon carcinogenesis preceding tumor development may be investigated. Such studies could provide valuable insight towards identifying cellular changes occurring in the early stages of carcinogenesis that may indicated an increased or decreased potential for tumor development in the later stages of colon carcinogenesis.

The hypothesis of this dissertation is **that ER will exert different effects on the growth of preneoplastic lesions depending on their phenotypic features. This effect is mediated via direct energy deprivation during specific stages of colon carcinogenesis and/or through alteration of biochemical events involved in cell growth and differentiation.** Based on this hypothesis, the main objectives of this dissertation were: (a) to evaluate the effect of ER in low and high fat diets on the stepwise development of colon cancer; and (b) to assess the effects of ER on the biochemical and cellular events related to cell growth and differentiation.

In concert with these two broad objectives, seven studies were designed to systematically test the hypothesis. The specific objectives of each study were to assess the effects of ER:

(a)   on enzymatic parameters associated with cell growth and differentiation in high

and low fat diets in young and old rats in non carcinogen treated colonic mucosa (Chapter 4);

(b)   at two levels (20% and 40%) in high fat diets on cellular enzymatic and proliferative events in non carcinogen treated colonic mucosa (Chapter 5);

(c)   in low and high fat diets on the phenotypic characteristics of ACF in the early and intermediate stages of carcinogen treated colonic mucosa (Chapter 6 and 7);

(d)   in low and high fat diets, in conjunction with intervention of the disease process at a late time point, on the number and growth characteristics of ACF, microadenomas and tumors (Chapter 8) and on enzymatic parameters in colonic mucosa and tumors at the later stages of colon carcinogenesis (Chapter 9).

(f)   analyses of morphological determinants of different stages of colon carcinogenesis at the molecular level (Chapter 10).

2. REVIEW OF THE LITERATURE

2.0 Cancer

2.0.0 Cancer Definition

"What gets cancer-the genes, the cell, the organ, the organism?

Perhaps even the population?" (Potter, 1993).

Cancer is a heterogeneous disease whose origins are the focus of thousands of investigations, yet the disease itself escapes definiticn.   A less futile approach may be to simply refer to cancer as a group of diseases that display some level of disorder. As stated by Potter, even the origins are of a multi-level nature. It may be more appropriate to embrace a multi-level, multi-faceted approach in order to understand the nature of the million and one diseases referred to as "cancer".

2.0.1 Multistage process of carcinogenesis

Delay in the development of tumors (Rous and Kidd 1941) , and the appearance of noncancerous lesion (Farber and Cameron, 1941) prior to the appearance of the tumor, have shaped the way carcinogenesis is viewed. Carcinogenesis is currently viewed as a multistage process induced by chemical carcinogens (Farber, 1984, Weinstein, 1992) involving the induction of multiple genetic and epigenetic events in cells which further undergo clonal expansion due to a selective growth advantage (Harris, 1991).

Carcinogenesis has been broken down into three definable stages, however each stage itself represents a complex multi step process. The first stage is defined as

initiation and involves the exposure of a cell to a carcinogen which can be chemical, viral or physical (such as radiation) in nature resulting in changes at the genetic level. Such alterations result in a selective growth advantage and altered responsiveness to inter-and extracellular signals (Harris, 1991)

The second stage of cancer development referred to is considered to be a promotional phase. Promotion involves the selective clonal expansion of the initiated cells into identifiable focal lesions (Farber, 1984; Harris, 1991). It has been proposed that these initiated cells remain latent until they are exposed to promoting agents (Franks, 1986). Tumor promoting agents are not inherently carcinogenic but may act to induce cells to divide in a initiated tissue (Franks, 1986). They may also reduce the time required for tumor development when given following a low dose of carcinogen (Weinstein, 1988).

Progression is the third stage of carcinogenesis and refers to phenotypic changes resulting in the development of malignancy and eventual metastasis (Harris, 1991). Malignancy refers to the potential of a lesion to metastasize (Liotta, 1992) and is identified by the presence of cellular abnormalities and invasion of surrounding tissues (Franks, 1986). Metastasis is defined as the transfer of disease from one organ or part to another not directly connected with it and may be due to the transfer of pathogenic organisms or cells as in malignant tumors (Hart, 1986). The tumors that develop are sites in the body distant from the primary tumor are considered to be secondly tumors (Liotta, 1992).

2.1   Colon Carcinogenesis

2.1.0 Normal colon (structure)

The colon consists of 4 layers termed the mucosa, submucosa , muscularis

externa and the serosa (Lev, 1990). The mucosa consists of vertical crypts arranged

perpendicular to the muscularis mucosa and intervened with the connective tissue

lamina propria (Lev, 1990). These test-tube like crypts are referred to as the crypts of

Lieberkun (Lev, 1990). Colonic crypts are predominantly made up of goblet, and

vacuolated absorptive, and enteroendocrine cells (Change and Leblond, 1971).

However, it is thought that all three cell lineages originate from a common stem cell at

the base of the crypt (Kirkland, 1988).

Colonic cells migrate from the bottom to the top of their crypt, attaining varying

levels of differentiation. The lower one third is lined with immature dividing cells and

is considered the normal proliferative compartment (Simanowski et al., 1989). On

migration toward the upper portion of the crypt, cells undergo terminal differentiation

and transform into columnar and goblet cells. Cells at the top third of the crypt are

fully differentiated, nonproliferating cells (Chang, 1984).

2.1.1 Cancer of the Colon

Colo-rectal cancers have been identified as one of the major sources of

morbidity and mortality in most western populations (Miller et al., 1983). Colon

cancer is reported to be the second leading cause of death among cancers (Schatzkin et

al., 1996). Human colon cancer occurs in the ascending, transverse and descending

areas of the colon (Fry et al., 1989) and may be classified by tumor syndromes

(Fearon, 1995). These syndromes are classified by the location and morphological

characteristics, along with the identifiable genetic alteration (Fearon, 1995). Several

risk factors for colon cancer have been identified including heredity factors such as

family history of colon cancer, genetically transmitted disease (familial polyposis,

Gardner's syndrome, inflammatory bowel disease), increasing age and diet (Fry et al.,

1989). Dietary factors identified as having an influence on the development of colon

cancer in humans will be discussed later. Colonic mucosa possesses one of the largest

interfaces between humans and the external environment and it is proposed that through

the removal of water in the intestine, the concentration of chemical exposed to the

mucosa is greatly enhanced (Pretlow, 1994). Therefore, it is not surprising that

patients diagnosed with colon cancer usually have more than one identifiable lesion

(Moertel et al., 1958).

Colon cancer is of an epithelial origin (Hermanek and Karrer, 1983) with 7

types of identifiable lesions including; adenocarcinoma; mucinous adenocarcinoma;

signet-ring cell carcinoma; squamous cell carcinoma; adenosquamous carcinoma;

undifferentiated carcinomas; and unclassified carcinoma and has been reviewed

(Morson and Sobin, 1976). The most common type of lesion is the adenocarcinoma

which exhibits glandular structural characteristics (Lev, 1990) that can be villus in

nature (Fearon, 1995). Such adenocarcinomas also retain identifiable colonic crypt

structures (Fearon, 1995).

2.1.2 Histogenesis of colon cancer

Treatment of the colon with a carcinogen results in a sequence of initial events involving alterations at the genomic level followed by changes in DNA synthesis and mitogenesis (Chang, 1984). As a result of these genetic aberrations, several identifiable morphological changes arise. Several dysplastic changes accompany colonic preneoplasia and neoplasia in the colon including; varying degrees of hyperchromatic staining, pseudostratification (with and without loss of polarity), increased mitotic figures, enlarged nuclei with prominent nucleoli; cytoplasmic basophilia; high nuclear to cytoplasmic ratio; and changes in goblet cell differentiation.(Chang, 1984; Deschner, 1974; Pretlow, 1994). Normal colonic epithelium is polyclonal and arises from numerous stem cells (Fearon, 1990). In contrast, it has been proposed that colonic neoplasms are monoclonal and develop from single dysplastic crypts which arise through repopulation of normal crypts by stem cells that have undergone carcinogen induced mutations (Bussey, 1975;Chang, 1984). Therefore, the evolution of an adenoma may stem from a single dysplastic crypt which proliferates to form many crypts all exhibiting dysplastic features (Bussey, 1975).

Abnormalities in tissue architecture distinguish dysplastic crypts from normal surrounding populations including; dilation, branching, changes in mucins (Baril et al., 1990; Shamsuddin et al., 1985); increased diameter and invagination of the epithelial lining (Chang, 1984; Deschner et al., 1988). Changes in cell proliferation (Lipkin, 1983; Lipkin and Deschner, 1976; McGarrity et al., 1988) and altered enzymatic activity (Barrows et al., 1990) have also been identified in dysplastic crypts. Whether

or not a colonic crypt must obtain all, or certain subsets of these identified anomalies in order to progress to a higher state of preneoplasia is not known.

Similarly, the exact nature of the histogenesis of adenocarcinomas is controversial (Shamsuddin et al., 1985). One proposal suggests that colorectal cancers arise from benign adenomatous polyps called adenomas (Muto et al., 1976) and are part of the "adenoma-carcinoma sequence" coined by Jackman and Mayo (1951). This theory has been further supported by recent studies demonstrating that the removal of polyps from human mucosa significantly reduce the risk of cancer (Murakanni et al., 1990) as well as by the development of cancer in individuals diagnosed with syndromes that strongly predispose them to the development of hundreds of adenomas (Burt and Samowitz, 1988). Thus adenomas are accepted as precursor lesions of carcinomas (Lev, 1990). The malignant potential of an adenoma may be assessed with respect to its size, villous development, and degree of dysplasia (Konishi and Morson, 1982; Riddell et al., 1983).

An opposing theory termed the " *de novo*" hypothesis suggest that cancer may evolve directly from flat mucosa without a prior adenoma stage in rats (Maskens and Dujardin-Loitus, 1981). The evidence to support this theory in human mucosa is not well established. However cancer is an asymptomatic disease for most of its life span, and once identified at the later stages of disease, it is hard to prove that the lesion did or did not evolve initially from an adenoma (Fenoglio and Lane, 1974). Consequently, the significance of this pathway in the histogenesis of colon cancer continues to be debated (Bedenne et al., 1992).

2.1.3 Proposed genetic model for colon cancer

With the advent of powerful molecular biological techniques over the last fifteen years, the idea of a genetic basis for cancer has been substantiated (Fearon and Jones, 1992) and estimated to account for more than 10 per cent of colorectal cancers (Utsunomiya and Lynch, 1990). Both inherited and somatic (arising in non-germ cells during the individual's lifespan) mutations have been identified in human cancers (Fearon and Jones, 1992). In particular, colon cancer is continually referred to in the scientific literature as an ideal model for the study of the nature, role, and origins of both types of mutations because of the natural history and inherited syndromes of predisposition to cancer development (Fearon and Jones, 1992; Vogelstein et al., 1988; Vogelstein et al., 1989; Weinberg, 1989). Colon cancer is unique in comparison to cancer of other tissues in that many types of tumors of varying stages of malignancy may be obtained for study (Fearon and Jones, 1992).

It is currently hypothesized that the genetic damage in colorectal cancers is of two lineages, the dominant oncogene and the recessive tumor suppressor gene (Bishop, 1991; Cooper, 1992; Fearon and Vogelstein, 1990; Levine, 1995). Oncogenes originate from normal cellular genes, termed proto-oncogene, as a consequence of genetic alterations resulting in abnormal gene expression , or the synthesis of altered proteins (Cooper, 1991). Proto-oncogenes encode proteins involved in signal transduction pathways and cellular transcription factors (Cooper, 1991, Levine, 1995). It is currently believed that the genetic alteration of a proto-oncogene , termed oncogene after conversion, results in a "gain in function" lending towards abnormal or

enhanced growth of the cell (Cooper, 1991, Fearon and Jones, 1992, Levine, 1995, Vogelstein, 1987). Tumor suppressor genes encode proteins involved in regulation of growth and differentiation in a negative fashion and may indirectly suppress neoplastic development (Knudson, 1985). Thus allelic losses (loss of heterozygosity) are believed to result in "a loss of function" of the tumor suppressor gene (Knudson, 1985, Levine,1995).

Vogelstein and Fearon (Fearon and Jones, 1992, , Fearon et al., 1987, Fearon er al., 1990, Vogelstein et al, 1988, Vogelstein et al., 1989, Vogelstein and Fearon, 1990) have discussed and reviewed the foundations of the genetic model for colorectal tumorogensis. This model acknowledges the multistage nature of colon cancer development and suggests that colorectal tumors arise as a result from the mutational activation of oncogenes coupled with mutation inactivation of tumor suppressor genes. They further hypothesize that mutations in at least 5 genes are necessary for the formation of a malignant tumor and that it is the total accumulation of mutations, rather than the order in which they occur, which determine the biological properties of the tumor. This model has also related the specific chromosomal alteration and its location to varying stages of phenotypic development from normal epithelium to a carcinoma and is detailed in Figure 2.1. It is important to note that the significance of the various inherited and somatic mutation to the cancer cell phenotype has not been identified. This model merely provides a foundation for the genetic nature of cancer development. There is little understanding of the relationship between dietary and environmental agents associated with increased risk of colorectal cancer and the

Figure 2.1 The proposed genetic model for colorectal tumorigenesis (Fearon and Vogelstein, 1990). The progression of tumorigenesis through a series of genetic alterations involving the RAS oncogene and tumor suppressor genes familial adenomatous polyposis (FAP), deleted in colorectal cancer (DCC) and p53. Transition from normal epithelium to invasive carcinoma is matched with particular chromosomal alterations. However, the authors caution that the order of these changes is not invariant and the overall accumulation, rather than the order in which they occur, may be the most important factor in tumorigenesis.

CHROMOSOME   5q   12p   18q   17p
ALTERATION MUTATION OR LOSS   MUTATION   LOSS   LOSS
GENE   FAP   K-RAS   DCC?   p53

DNA
HYPOMETHYLATION

OTHER
ALTERATIONS

| NORMAL EPITHELIUM | → | HYPERPROLIF EPITHELIUM | → | EARLY ADENOMA | → | INTERMEDIATE ADENOMA | → | LATE ADENOMA | → | CARCINOMA | → | METASTASIS |
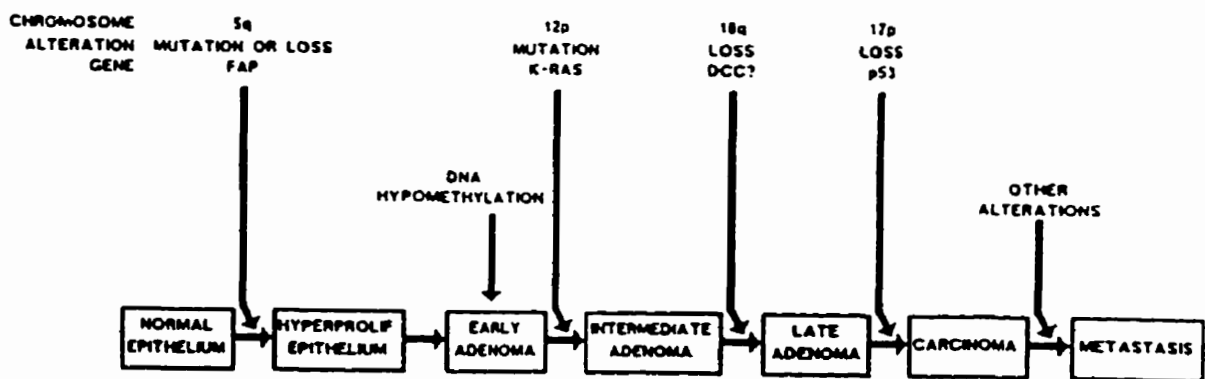
mutational events seen in normal and tumor cells.

2.2. Energy Restriction (ER)

2.2.0 What is ER?

There is no definitive description of ER in the literature, which may be the result of variances in its application to an experimental protocol. ER has been induced simply by; reducing the time of exposure to food (Duffy et al., 1989); reducing the amount of one (Kumar et al., 1990) or more than one macronutrient (Birt et al., 1993). ER has also been introduced in conjunction with a reduction (Lok et al.,1987) or maintenance of vitamins and mineral (Birt et al., 1992, Kumar et al., 1990, Reddy et al., 1987). Variances are also noted in the time of introduction and length of exposure to ER (Kritchevsky, 1993). Despite the variances these investigations commonly reduced the intake of energy in experimental groups compared to the level of energy consumed freely by the control groups. Initial studies employing ER used the term "caloric restriction". However, recent investigations have adopted the term ER as a more specific term indicating the reduction is in fact in the level of energy in the diet.

2.2.1 ER and age, longevity

As far back as fifteenth century ER has been associated with increased longevity (Butler, 1918). Luigi Coronora reportedly led a life of excess until the age of 40 and thereafter subsisted on only 14 ounces of food per day, plus wine and exercise. Consequently after attaining the ripe old age of 100, he became known as the "Venetian Centenarian" (Weindruch et al., 1991). The first experimental study employing ER in rodents was conducted by McCay and Crowell (1934) who demonstrated that rats

subjected to ER lived to an average of 850 days as compared to 480 days of their fully

fed counterparts. Within the last decade, there has been a resurgence of interest in the

ability of ER to significantly retard the onset and the physiological changes associated

with aging. Aging has been defined as a decline in the morphologic integrity and

functional capacity of organs and tissues associated with a decline in ability to maintain

homeostasis (Meites, 1990). In an attempt to understand the possible mechanism(s) by

which ER diminishes the effects of aging several investigations have been conducted in

rat models (Breese et al., 1991, Duffy et al., 1989, Hass et al., 1992, Kalant et al.,

1988, Leakey et al., 1989, Umezawa et al., 1990). The result of such studies has lead

to the development of several theories at the mechanistic level including the ability of

ER to diminish the effect of aging on drug metabolizing enzymes (Leakey et al., 1989),

circulating hormones (Meites, 1990) and immunological functions (Umezawa et al.,

1990, Fernandes, 1995). Such theories may in part explain the ability of nutrition (in

this case energy) to influence the aging process.

2.2.2 Physiological effects of ER

ER has been the focus of many animal and to a lesser extent human,

investigations. These investigations have substantiated the ability of ER to significantly

affect a plethora of physiological parameters. Most of the investigations in humans

are confounded by the inability to control for all experimental variables. Despite such

hurdles ER in humans has been shown to affect several biological parameters including;

the sympathetic nervous system (Eliahou et al., 1992), insulin sensitivity (Nakai et al.,

1992), sleep apnea (Suratt et al., 1992), body composition (Kreitzman, 1992), substrate

oxidation, (Franssila-Kallunki et al., 1992), calcium homeostasis (Nishizawa et al., 1992), lipoprotein and fatty acid metabolism (Parenti et al., 1992, Tang et al., 1993) and colonic cell proliferation (Steinbach er al., 1994).

The rat model provides an opportunity to conduct more extensive and encompassing studies in which the mechanisms of ER may be further elucidated. Parameters affected by ER in rat include; changes in resting metabolic rate and thermogenesis (Van Gaal et al., 1992); decreased fat stores in the body (Kumar et al., 1990); decreased body temperature (Duffy et al., 1989; adrenocorticotropic hormone, prolactin, corticosterone, thyroid stimulating hormone (Armario et al., 1987; Pariza et al., 1987); serum growth factor levels and their binding capacity (Breese et al., 1991; Hursting et al., 1993; Rugerri et al, 1989a, 1989b); enzymes involved in glucose metabolism (Kalant et al., 1988); changes in P-450 isozymes, mixed function oxidase system (drug metabolizing enzymes) (Leakey et al, 1989; Hashmie et al., 1989); enzymes involved in free radical formation (Rao et al., 1990; Walfrod et al., 1987); cell proliferation in various tissues (Lok et al., 1988); immune response (Umezawa et al., 1990; Fernandes et al., 1995); mRNA expression (Koizumi et al., 1990; Spindler et al., 1990); cellular oncogene expression (Baik et al., 1992); DNA polymerase activity (Srivastava et al., 1992); and enzymes involved in cell growth and differentiation (PKC) and signal transduction (TK) (Birt et al.,1993; Craven and DeRubertis, 1992; Kumar et al., 1990).

ER has also been demonstrated to significantly modulate the development of chemically induced cancer in several tissues such as ; mammary (Pollard and Luckert,

1985); pancreatic (Roebuck et al., 1993); skin (Birt et al., 1993); liver (Lagopoulos et al., 1992; Sugie et al., 1993); lung (Tannenbaum, 1945); lymph (Weindruch et al., 1986) and colon tissues (Pollard and Luckert, 1985; Klurfeld et al., 1987; Kumar et al., 1991; Reddy et al., 1987)

Clearly, ER provides a modem in which several factors may work together to modulate an organism's response to its surrounding environment. Therefore, it is not likely that one mechanism *per se* will be identified as the sole reason for an observed effect of ER on physiological event (s) *in vivo*.

2.3   Modulation of Colon Cancer - Experimental Approaches

2.3.0 Epidemiological Studies

There is substantial epidemiological evidence to support a role of diet in the development of colon cancer (Doll, 1992; Gorbach and Goldwin, 1990; Vogel and McPherson, 1989; Weisburger, 1989). Over the past few decades, the relationship between colorectal cancer and dietary habits has been studied extensively with the aim of identifying components of a diet (types of foods, vitamins, minerals, macronutrients) associated with increase or decreased risk (Doll, 1992; Neuget et al., 1991; Potter et al., 1993). A culmination of such data suggests that some aspect of a diet high in, meat, fat, protein, total energy and low in fibre, vitamins and minerals are important in the etiology of colon cancer. A recent study has also provided evidence to support that diversity (variety of food consumed) may also be related to a moderately decreased risk in colorectal cancer (Fernandes et al., 1996).

The current method of measurement for associated risk pertains to the

reoccurrence, regression or conversion of adenomatous polyps into adenocarcinomas (Lanza et al., 1996; Little et al., 1993; Potter, 1993; Schatzkin et al., 1996).

As mentioned in the previous section, most of the epidemiologic studies focus on a variety of dietary factors, more notably fat intake, and do not directly associate the amount of food/energy in the to cancer risk. Such confounding factors may be the balance among energy consumption , retention, expenditure and sources of energy (Pariza, 1987).

Epidemiological studies attempting to determine the relationship between caloric intake and increased risk of colon cancer are insightful yet at the present time inconclusive. Most studies have shown a positive association with energy intake (Jain et al., 1980; Bristol et al., 1985; Graham et al., 1988, Willet, 1989). However obesity appears to be weakly related to colorectal cancer (Willet, 1989). Interpretation of this weak association may be confounded by such factors as weight loss as a result of the disease (Little et al., 1993) the balance among energy consumption , retention, expenditure and sources of energy (Pariza, 1987). Consequently it has been suggested that the body mass index (as an indicator of obesity) is not associated with increased risk, whereas physical activity as an indication of energy expenditure is associated with a decreased risk (Little et al., 1993).

Culmination of this epidemiological data indicates a possible relationship between caloric intake and the genesis of cancer and provides the impetus for animal model studies in which confounding factors can be controlled and the etiology of cancer can be manipulated and explored to a greater extent.

2.3.1 Animal models

Animal models provide a means by which the modulation of colon cancer by diet can be examined at several different levels that are often unable to be assessed in human studies. However, it is important to note differences in experimental variables that may affect the results of animal model studies in colon carcinogenesis (Bird et al., 1985; Bird et al., 1996; Hamilton, 1989). The type, dose, route and schedule of administration of a carcinogen may produce variable results. Diet composition, the amount of consumption and the time of intervention (during carcinogen treatment, immediately after carcinogen treatment, or several months after carcinogen treatment) could result in very different effects on the initiation, promotion or progression of the disease. Various endpoints may be employed to measure the effects of a particular treatment including animal survival, enzymatic measurement, and various tumor characteristics. Concluding results of a particular experimental protocol may be dependent on the nature of the endpoint utilized. Cross-comparison of several studies should be in conjunction with acknowledgement of such experimental factors.

2.3.1.0 Colon carcinogens

The advent of chemicals capable of inducing colon cancer has fostered the study of colon carcinogenesis in rodents which rarely develop the disease spontaneously. The two most commonly used chemicals known to induce colonic neoplasms in rodent are 1,2-dimethyl hydrazine (1,2-DMH) and its metabolite azoxymethane (AOM) (Greene et al., 1987). Consequently, the neoplasms induced by these compounds share similar tissue architecture, histochemistry, and enzymatic characteristics with human

adenocarcinomas and therefore considered to be appropriate models for the study of colon carcinogenesis (Bird et al., 1985). AOM is the carcinogen repeatedly used in this dissertation, therefore the discussion of metabolism will be limited to this particular carcinogen.

2.3.1.1 Metabolism of AOM

Steps in the metabolism of AOM are detailed in Figure 2.2 (Fiala, 1977). AOM is metabolized to methylazomethanol (MAM) by alpha-hydroxylation (Green et al., 1987) in the liver. MAM may then be transported to and metabolized in extra hepatic tissues , such as the colon, or it may undergo further metabolism in the liver. The resulting metabolite is methyl diazonium ion and is the reactive alkylating agent (Weisburger, 1971) and may finally form methyl carbonium ions. These ions are thought to be capable of methylating macromolecules such as DNA (Fiala, 1977). A subcutaneous injection of AOM results in colonic neoplasms, however its isomer, N-nitroso-dimethylamine, is suggested to be a liver and kidney carcinogen (Sohen et al., 1991). Stem cells are believed to the ultimate target for carcinogenic mutations in the colon (Change, 1984; Potten, 1992).

2.3.1.2 Species and strain differences

Differences in susceptibility of tumorigenesis among species and strains of rats has been reported (Naito, 1981, Nauss et al., 1987; Nigro, 1985). Sprague-Dawley rats are considered to be more susceptible than F344 (Nauss et al, 1987), however F344 are a more inbred strain.

Figure 2.2 Metabolism of the colon carcinogen azoxymethane (AOM) (Fiala,

1977)

$$CH_3 - N = N - CH_3$$

$$\downarrow$$

$$O$$

Azoxymethane (AOM)

$$\downarrow$$

$$CH_3 - N = N - CH_2OH$$

$$\downarrow$$
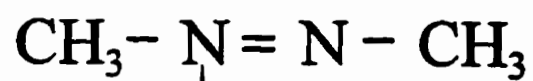
$$O$$ Methylazoxymethanol (MAM)

$$\downarrow$$

$$CH_3 - N \equiv N^+ \ OH^-$$

Methyldiazonium

$$\downarrow$$

$$CH_3^+$$

Methylcarbonium ion

2.3.2 Endpoints

2.3.2.0 Tumors

Tumor presentation is the most common benchmark used to signify the endpoint of colon cancer (Hamilton, 1989). Tumor development is indicative of disease and is important in assessing the potency of cancer inhibition or promotion. However, cancer is a multi stage disease and the sole use of tumor incidence as the endpoint does not allow for analysis of a potential cancer modulator at the varying stages of cancer. In addition, tumor presentation is a time-consuming, expensive and lengthy process (Bird et al., 1989). The etiology of the disease process may be further elucidated by comprehensive studies examining the strengths and weaknesses at several stages of the disease.

2.3.3 Biomarkers in the study of colon carcinogenesis

Biomarkers (risk markers) represent a group of endpoints that are used to study modulators of colon cancer. Boone et al. (1992) have suggested that a biomarker must fulfill a minimum of two requirements in order to be a potential intermediate endpoint. The biomarker must correlate with cancer risk and possess the ability to modulate towards normal by an effective chemopreventive agent (Boone et al., 1992). Cell proliferation, oncogene expression and enzymatic changes are all considered to be intermediate biomarkers and are used to assess cancer risk in the preneoplastic colon (Cooper, 1992; Lippman et al., 1990; Pretlow, 1994). The advantage of utilizing intermediate biomarkers stems from the shorter amount of time for development, thus studies employing these markers minimizes time and expenses (Bird et al., 1989).

However, the alterations observed in these markers may be an indication of malignant potential but may not actually be involved in the carcinogenic process (Pretlow, 1994). Therefore, there is a need to establish the validity of observed changes in these aforementioned intermediate biomarkers (Bird et al., 1989).

2.3.3.0 Cell proliferation

The use of cell proliferation as a biomarker for cancer has recently come into question. One stream of investigations suggests that the presence of stimulated cell proliferation without evidence of any other biochemical or morphological alterations is itself an indication of increased malignant potential (Ames and Gold, 1990; Cohen and Ellwein, 1990; Clayson et al., 1991). Conversely, the other side of the argument suggests that cell proliferation is only one factor of many that leads towards the development of neoplasia (Farber, 1995; Melnick, 1992; Weinstein, 1992). The strength of the latter argument resides in the observation of a lack of association between cell proliferation and cancer occurrence in several tissues and organs and the ability of genotoxic carcinogens to inhibit cell proliferation (Farber, 1995). In addition to this argument, it has also been postulated that rapid proliferation of cell may not be an indication of neoplasia (Toribara et al., 1989). From the technical side of this issue, the reproducibility of measured proliferative activity has also come into question. A recent study suggests that high variability exists from one lab to another when assessing the amount of "scorable tissue" for proliferative measurement (Roe et al., 1996). The stage of the cell cycle at which cell proliferation is measured may also be affected by the particular methodology employed in the investigation (Scalmati and Lipkin, 1992;

LaFave et al., 1994).

Altered cell proliferation has been identified in the entire human colonic mucosa exhibiting either an adenoma or adenocarcinoma (Lipkin, 1983;) and in those with ulcerative colitis (Beliberg et al., 1985; Biasco et al., 1990).   Pertaining to the rodent colon, administration of a carcinogen alters cell proliferation in histologically normal appearing crypts, which suggests that altered cell proliferation is an early event in colon carcinogenesis (Chan et al., 1976; Deschner , 1974; Lipkin and Deschner, 1976; Klurfeld et al., 1987). However, the value of cell proliferation as a valuable "predictor" in end stage tumorigenesis is not well established. Several studies have demonstrated that changes in cell proliferation do not correlate with end stage tumor development (Cameron et al., 1990; Galloway et al., 1986; Geltner-Allinger et al., 1991; Glickman et al., 1987; Kingsworth et al., 1986).

Moreover, it has also been postulated that changes in proliferative pattern of colonic mucosa due to diet modifications may not be predictive of modulation of end stage tumor incidence (Cameron et al, 1990). Consequently studies monitoring changes in cell proliferation by diet modification, such as ER and dietary fat, are conflicting in nature (Albanes et al., 1990; Bird and Stamp, 1986; Klurfeld et al., 1987; LaFave et al., 1994; Lok et al., 1988; Steinbach et al., 1993).

Nonetheless, measurement of cell proliferation remains one of the most commonly used biomarkers in cancer research. However as stated by Farber (1995) until a system is developed in which cell proliferation becomes the "only or at least a major variable under study" the use of cell proliferation as a risk factor must "remain in

the realm of unproven speculation".

2.3.3.1 Enzymes involved in cell growth and differentiation

Normal eukaryotic development is the result of a complex integration of several extra and intracellular events involving cell proliferation and differentiation. The signals that transduce throughout the cell proceed in a highly ordered manner (Hug and Sarre, 1993) and it is believed that neoplasia may be the result of significant alterations in this tightly regulated system (Cantley et al., 1991; Heldin and Westermark, 1994; Weinstein, 1987). Signal transduction pathways begin with a response to the binding of a ligand (hormone, growth factor, cytokine), considered to be a signal, to a membrane receptor resulting in a cascade of events ultimately resulting in modification of gene expression (Hug and Sarre, 1993).

There are several subsets of intracellular pathways which utilize some common and some exclusive targets in transduction of their message towards the nucleus. Phosphorylation and dephosphorylation of proteins is the major mechanism by which the signal travels within the cell (Hunter, 1987; Tonks and Charbonneau, 1989). Two major targets of phosphorylation are the serine/threonine and tyrosine residues throughout the cell. The family of enzymes termed protein kinase C (PKC) represent kinases which phosphorylates serine/threonine residues on targeted cellular substrates (Blobe et al, 1994; Hug and Sare, 1993). Tyrosine kinase is a general name used to describe several enzymes which phosphorylate tyrosine residues and may be further classified as receptor tyrosine kinases (RTK or non-SRC) and non receptor tyrosine kinases (SRC) (Hunter and Cooper, 1988; Ullrich and Schlessinger, 1990; Yarden,

1988).   Incidentally, the two families of enzymes have been demonstrated to exhibit integration or "cross-talk" (Radinsky, 1993;Ullrich and Schlessinger, 1990; Weismuller and Wittighoffer, 1994).

2.3.3.2 Protein Kinase C (PKC)

PKC was initially discovered by Nishizuka and colleagues (Inoue et al., 1977) in the rat brain.   They demonstrated its activity to be affected by limited by proteolysis, calcium (Takai et al, 1979), phospholipids (Kishimoto et al., 1980) and phorbol esters (Castagna et al, 1982).   Initial investigations characterized the enzymatic regulation of PKC assuming it was a single enzyme.   However, after cloning PKC cDNAs, it became apparent that PKC is a family of products of distinct genes representing closely related isozymes of PKC.   To this date the scientific literature demonstrates evidence of twelve different cloned PKC isozymes (Dekker and Parker., 1994).   These isozymes can be further classified into groups according to similarities in structure. Consequently, the differences in structure among the groups results in varying requirements for cofactor activation (Figure 2.3).   The structure of PKC consists of a single polypeptide chain containing regulatory and catalytic domains (Hug and Sarre, 1993).   All isozymes contain the catalytic region at the COOH terminal of the polypeptide chain, the variances occur in the regulatory domains.   The classical PKCs, α , $\beta 1$, $\beta 11$ and γ contain all regulatory regions and thus are activated by calcium, diacylglycerol and phorbol esters (Blobe et al., 1994).   The novel PKCs , δ, ϵ, and θ lack the domain referred to as C2 which contains the calcium binding site, thus these novel forms are calcium independent (Kaibuchi et al., 1989).   Atypical PKCs include

$\zeta, \iota, \lambda, \mu$ and η and lack the C2 and part of the C1 domain and therefore are calcium, DAG and phorbol ester independent (Hug and Sarre, 1993). Consequently, the CI domain consists of 2 zinc finger proteins responsible for phospholipid and phorbol ester binding (Kaibuchi et al., 1989). Some of the atypical forms demonstrate a lack of binding affinity in these regions or, as demonstrated for PKC ζ, possess only one zinc finger (Ono et al., 1989). It has been proposed that once the constant domains in the regulatory subunit are activated, the polypeptide undergoes a conformation change that opens the catalytic domain (Weinstein, 1987). Autophosphorylation, as a mechanism of activation, has been demonstrated and reviewed in several studies (Hug and Sare, 1993).

The tissue and cellular distribution of PKC isozymes has been reviewed (Wester at al., 1992). It is evident that PKCs are widely distributed in several types of tissues and in various organs. However, there appears to be quite a variation in the intracellular distribution between isoforms suggesting divergent roles for the isoforms within a tissue (Hug and Sare, 1993).

In reference to activation and subsequent translocation of PKCs, one common theory persists throughout the literature (Blobe et al., 1994, Hug and Sarre, 1993, Morrison et al., 1990; Schlessinger, 1986) and is partially depicted in Figure 2.4. It is widely accepted that PKCs are mainly located in the cytosol. Upon binding of a growth factor to their respective receptor, their tyrosine kinase activity directly or indirectly actives an enzyme called phospholipase C (PLC) which serves to cleave phosphatidyl inositol bisphosphate (PIP_2) into inositol tri-phosphate (IP_3) and DAG and

increases intracellular calcium. Calcium and DAG are thought to account for the

translocation and subsequent activation of PKC from the cytosol to the membrane. It is

widely believed that the membrane fraction is in fact the active form.

A review of the literature reveals a large number of putative substrates in the

PKC signal transduction pathway some of which are detailed in Figures 2.4 and 2.5.

Figure 2.3 Classification of PKCs according to structure (Transduction Laboratories ,

Lexington, Kentucky).

Protein Kinase C

Classification of the known PKCs according to structure

Classical PKCs (cPKC) - alpha, beta, gamma

New PKCs (nPKC) - delta, epsilon, theta

Atypical PKCs (aPKC) - zeta, iota, lambda, mu
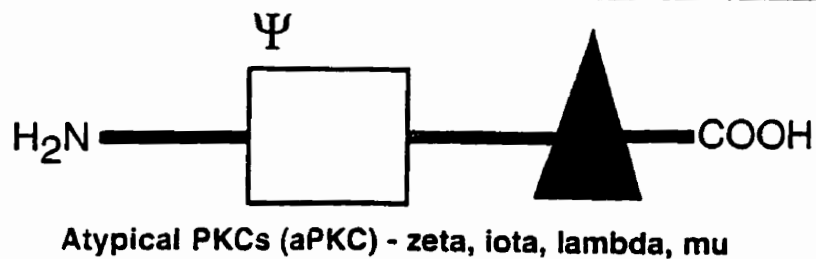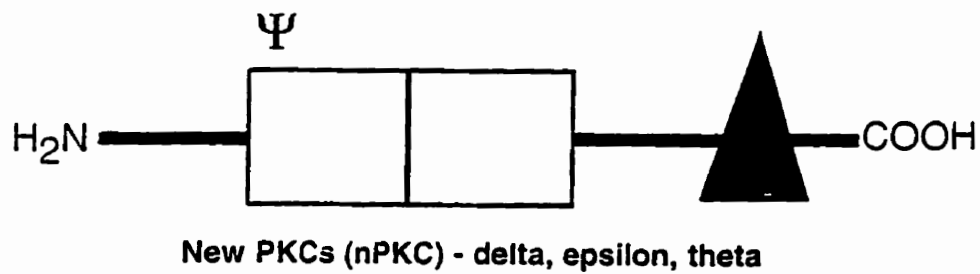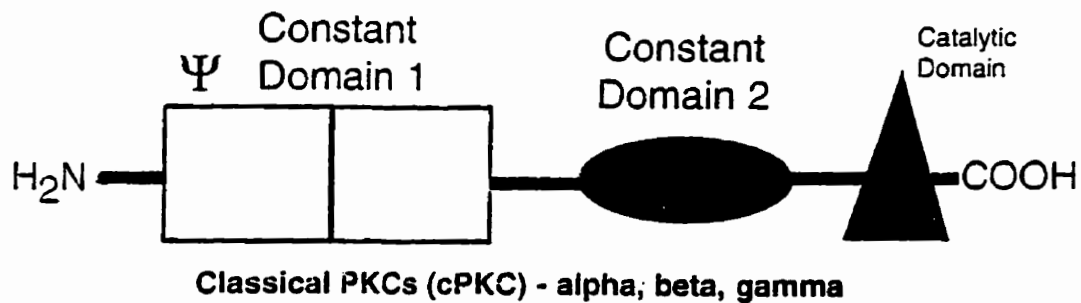
Three major categories of substrates emerge including; proteins involved in signal transduction and PKC activation (epidermal growth factor , T cell and insulin receptors, RAS and GAP); proteins in metabolic pathways; and proteins involved in regulatory functions in gene expression (transcription and translation factors) (Hug and Sarre, 1993).

Potential roles of PKC in several types of cancers has been substantiated (Blobe et al., 1994) however, relevant to this dissertation, the role of PKC in colon cancer will be reviewed. The interest in PKC as a colonic tumor promoter emerged from the observation of PKC activation by tumor promoting phorbol esters (Castagna et al., 1982). In addition, the activation of PKC by lipid (Bell and Burns, 1991) and bile acids (Craven and DeRubertis, 1986) both of which are present in the colon provided impetus to explore the role of this enzyme in both human and rodent colon.

Several investigations have demonstrated that colonic adenomas and adenocarcinomas in humans (Attar et al., 1996; Guillem et al., 1987; Kopp et al., 1991; Kusunoki et al., 1992;Levy et al., 1993) and in rats (Craven and DeRubertis, 1992; Nelson and Holian, 1991) exhibit down regulated levels of PKC activity as compared to normal and adjacent mucosa. This relationship held true for total (membrane and cytosol), and membrane and cytosol fractions individually. Such an observation seem paradoxical in nature. As discussed previously, PKC is enhanced by known tumors promoters. However, it has been proposed that PKC may be activated initially by such substances which may eventually lead to a prolonged down regulation (Blobe et al., 1994).

Figure 2.4 Proposed model for PKC activation and translocation to the membrane

(Radinsky, 1993). Abbreviations include; GF (growth factor); PLC (phospholipase

C); PI (phosphatidyl inositol); $P1-4,5-P^2$ (phosphatidyl inositol bis phosphate);

IP^3(inositol tri-phosphate); DAG (diacylglycerol); PKC (protein kinase C).
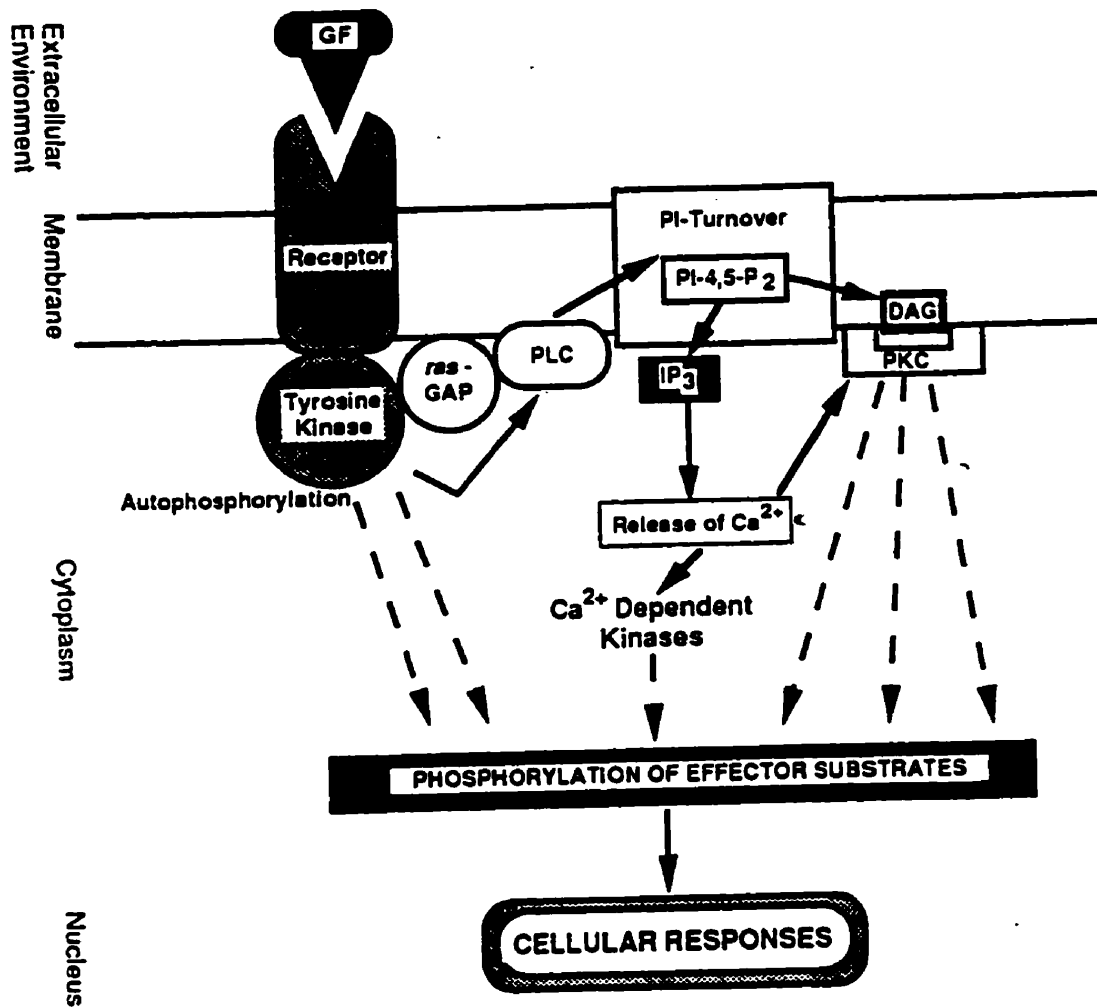
Figure 2.5 Interaction of PKC in cellular functions (Cantley et al.,1991).

Abbreviations include; PLC (phospholipase C); PI (phosphatidyl inositol); P1-4,5-P^2

(phosphatidyl inositol bis phosphate); IP^3(inositol tri-phosphate); DAG (diacylglycerol);

PKC (protein kinase C).

RECEPTOR
TYROSINE KINASE
▼
AUTOPHOSPHORYLATION

(RECRUITMENT OF CYTOSOLIC ENZYMES TO MEMBRANE)

PRIMARY
RESPONSES

ras-GAP

c-raf

PI PLC-γ   p74   PI 3-KINASE   OTHER
                                                      SUBSTRATES
                                                      CALPACTIN
                                                      EZRIN

GTP*ras* | GAP-pp190?
         | GAP-pp62?

DAG   I-1,4,5-P3   PROTEIN-SER   PI-3-P
                        PHOSPHORYLATION PI-3,4-P2 PI-3,4,5-P3

(EFFECTOR?)

        ?
                        Ca^{2+}↑

RECEPTOR
    PATCHING?
ION CHANNELS?
TYR
KINASES?
LIPASES?
DAG
ARACHIDONATE

Ca^{2+}

PKC

?   ?   ?

                                                 Ca^{2+}

TYR KINASE   ACTIN FILAMENT
                               REARRANGEMENT
pp42
(MAP KINASE)   MEMBRANE RUFFLING?
                               CHEMOTAXIS?
S6 KINASE   CELL ROUNDING?
                               CELL DIVISION?
RIBOSOMAL
S6 SUBUNIT

Na/H
EXCHANGE

pH↑ Na/K ATPase

K+↑

PROTEIN
SYNTHESIS

NF-ΚB: IΚB
(*rel*)

PHOSPHATASE?

NF- ΚB   DEX
(*rel*)   RECEPTOR ⇌ *jun*-P-THR   SRF
                                           SRE

                         PO4   *fos*

                               jun/fos ⟵······ E1A
                                (AP-1)
                                 TRE

NUCLEUS

In addition, decreasing level of DAG in cancer tissue may also serve to decrease PKC activity (Blobe et al., 1994). Conversely, it has been suggested that an observed increase in PKC activity may indicate a previously inactivated state, and that decreased activity indicated a previously activated state (Craven and DeRubertis, 1992). This argument suggests that PKC activity may be higher in rat and human neoplasms prior to measurement by assay conditions or that PKC activity could be open to more than one interpretation.

It has been argued that the methods employed to measure PKC activity vary considerably and that PKC itself may vary considerably from one human colonic neoplasm to another (McGarrity and Peiffer, 1994) which would question the use of PKC activity as a risk marker.

Immunocytochemical and analysis of PKC revealed the presence of α, δ, ϵ and ζ isoforms in rat mucosa (Jiang et al., 1995). Detection of the amount of the isozyme by Western Blotting techniques have revealed the presence of α, β, δ, ϵ, ζ and η (Craven and DeRubertis, 1994a, Davidson et al., 1994) in normal human mucosa and α, β, δ, ϵ, and ζ in rat mucosa (Craven and DeRubertis, 1992, Davidson et al, 1994). Presence of mRNA by RT-PCR reveals a similar pattern of isozyme expression (Davidson et al., 1994; Jiang et al., 1995).

Human adenocarcinomas have been reported to exhibit a higher amount of PKC β, δ, ζ, and η (Davidson, 1994) than normal mucosa. Rat colonic adenocarcinomas have been examined for the presence of α, β, and γ by immunoblotting techniques and have demonstrated the β form to be the predominant

isozyme (Craven and DeRubertis, 1992).

The sequential analysis of PKC isoform expression in normal, preneoplastic and neoplastic tissue within the same mucosa is lacking. However, colonic PKC activity has been shown to be significantly affected by dietary fat (LaFave et al., 1994) and fiber (Chapkin et al., 1993). ER has been demonstrated to significantly reduce PKC activity in skin (Birt et al., 1994) however, the effects of ER on colonic PKC has not been determined.

It is evident that PKC activity and distribution of isoform expression is altered in colon cancer in both the rat and human. However, the significance of increased activity, or changes in particular isozymes has not been elucidated.

2.3.3.3 *Tyrosine Kinases*

As mentioned previously, there are two types of tyrosine kinases. The transmembrane receptor family (non-SRC) and the cytosolic non-receptor family (SRC). Figure 2.6 (Cantley et al., 1991) depicts the location and function of both types of tyrosine kinases. Receptor tyrosine kinases undergo ligand induced dimerization, when a growth factor binds with its receptor, resulting in cross-phosphorylation of the subunits on the tyrosine residues. The resulting tyrosine phosphorylated regions provide binding sites for cytosolic enzymes with SH-2 domains. SH-2 domains are noncatalytic SRC homology regions that are conserved among a series of cytoplasmic signalling proteins regulated by receptor tyrosine kinases (Koch et al., 1991). Upon association, these proteins become substrates for the receptor protein tyrosine kinase. It is interesting to note that the activation of PLC

results in activation of PKC (Figure 2.4). Receptors demonstrated to be RTKs are

generally divided into three groups; the epidermal growth factor receptors (EGFR-TK);

platelet derived growth factor (PDGF-R); and the insulin receptor (I-R) (Yarden et al.,

1988).

It is evident, from Figures 2.4, 2.5 and 2.6 that SRC tyrosine kinases target the

tyrosine residues on phosphatidyl inositol 3-kinase (PI 3-Kinase) and RAS and GAP

proteins. However, the function and activation of the SRC kinases are less well

understood (Kypta et al., 1990). The SRC family of tyrosine kinases include; $p60^{c-}$

^{src}(SRC); $p61^{c-yes}$ (YES); $p56^{lck}$(LCK); $p59^{fyn}$ (FYN); $p59^{hck}$ (HCK); $p56^{lyn}$ (LYN); $p55^{c-}$

^{fgr} (FGR); and $p55^{blk}$ (BLK) (Park et al., 1993). The activation and repression of the

SRC family kinases has been proposed to be induced by changes in the activities of

specific kinases, phosphatase or allosteric effectors (Cooper and Howell, 1993).

Interestingly, the integration of the SRC and non-SRC tyrosine kinases have been

demonstrated with the PDFG-R and its activation of SRC, FYN and YES SRC tyrosine

kinases (Kypta et al., 1990). Consequently , the SRC and non-SRC are considered to

be part of the growth factor and transducer of growth factor responses categories

classifying them as potential oncogenes (Cantley et al., 1991).

Figure 2.6 SRC and receptor type tyrosine kinases and their potential substrates

(Cantley et al., 1991). Abbreviations include; PLC (phospholipase C); PI

(phosphatidyl inositol); P1-4,5-P^2 (phosphatidyl inositol bis phosphate); IP^3(inositol tri-

phosphate); DAG (diacylglycerol); PKC (protein kinase C); SH (SRC homology

regions).

"RECEPTOR TYPE"
PROTEIN-TYROSINE KINASE

"SRC TYPE"
PROTEIN TYROSINE KINASE
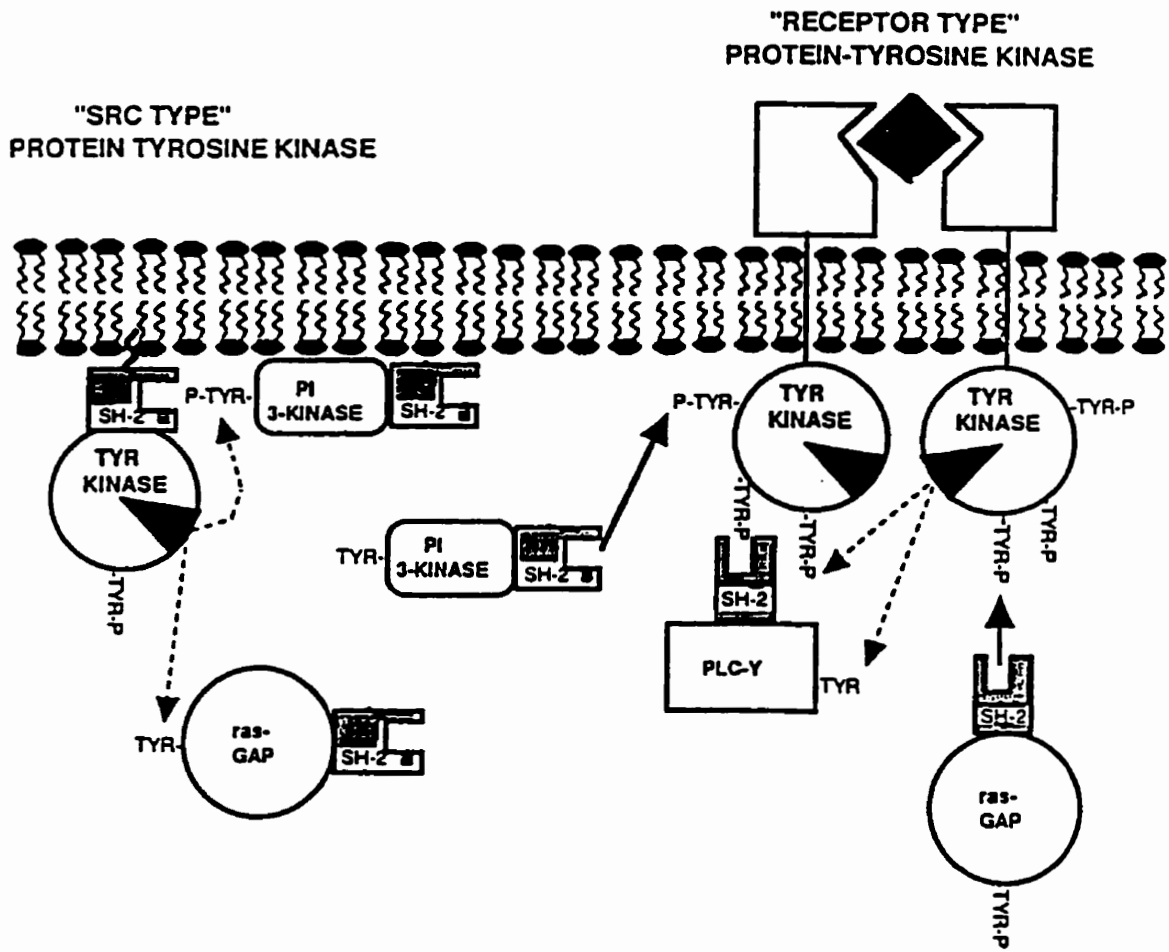
Tyrosine kinases have been implicated to play a role in the development of colon cancer (Arlow et al., 1989; Bolen et al., 1987; Carwright et al., 1989, 1990; DeSeau et al., 1987; Malecka-Panas et al., 1996; Sakanoue et al., 1991; Sing et al., 1992). In humans, elevated tyrosine kinases has been observed in neoplastic mucosa as compared to normal mucosa (Sakanoue et al., 199; Schwartz et al., 1990). In particular, elevated levels of SRC and YES have been demonstrated in colonic adenoma and adenocarcinomas (Bolen et al., 1987; Cartwright et al, 1989). Elevated level of SRC in premalignant tissue has also been demonstrated in epithelia of ulcerative colitis patients (Cartwright et al., 1994) suggesting that SRC is important in early stages of neoplastic development. The tyrosine kinase associated with EGFR (EGFR-TK) has been shown to be elevated in human colon carcinoma cells and has been suggested to be a predictor of metastatic potential (Radinsky et al., 1995).

In rat mucosa the majority of the investigations have employed a method of analysis which measures the activity of all tyrosine kinases without distinction between SRC and non-SRC activity. Carcinogen treatment of colonic rat mucosa results in elevated tyrosine kinase activity (Arlow et al., 1989; Kumar et al., 1990; Rao et al., 1993; Sing et al., 1992). A novel study which employed a unique method to measure specific EGFR-TK activity demonstrated an increase in activity in AOM treated colonic mucosa relative to non AOM treated mucosa.

Consequently, treatment of rat colonic mucosa with calcium (Arlow et al., 1989), piroxicam (a nonsteroidal anti-inflammatory drug) (Singh et al., 1992) and curcumin (a phenolic compound) (Rao et al., 1993) suggests a potential role for

decrease tyrosine kinase in the inhibition of neoplastic development. In relation to ER, tyrosine kinase has been demonstrated to be decreased by 20% ER in colonic mucosa and tumors in rats (Kumar et al., 1990). However, there the effects of the amount of dietary fat on tyrosine kinase has not been established.

As in the literature involving PKC, the significance of increased or decrease activity and expression of SRC and non-SRC tyrosine kinases is not definitive. Their worth as biomarkers should be established through further research involving the changes in these enzymes throughout the varying stages of carcinogenesis.

2.3.4 Precursor Lesions

As discussed previously, the utilization of risk markers as endpoints circumvents lengthy experimental duration and expense. Their limitation stems from the fact that they are not the disease itself and merely represent a marker for increased risk of disease development (Bird et al., 1989). The identification and utilization of precursor lesions of colon cancer would provide an ideal endpoint, having the advantage of being the actual biological lesion of interest, allowing for the stepwise assessment of the disease process, and a shorter experimental duration lending towards a decreased cost (Bird et al., 1989). Preneoplastic lesions and their subsequent importance in understanding the role of tumor promoters on the disease process have been elucidated in the liver model of carcinogenesis (Farber, 1984; 1986; Peraino et al., 1983). These lesions are in the form of altered enzyme hepatic foci of the liver (Farber, 1984). Within the colon, several putative preneoplastic lesions have been identified in colons of rodents treated with colon specific carcinogens (Barrow et al.,

1988, 1990; Cooke et al., 1984; Chang, 1984; Deschner, 1974; Preltow et al., 1990). There is a great deal of interest in preneoplastic lesions for their applications in chemoprevention studies (Kelloff et al., 1992). An ideal preneoplastic lesion has been suggested to possess ceratin qualities including; ease of measurement (non invasive techniques; appearance early in carcinogenesis; high sensitivity and accuracy relative to cancer; demonstration of modulation by chemopreventive agents; correlation of modulation with decreased cancer incidence (Kelloff et al., 1992). Incidentally, the use of all of the identified colon precursor lesions in the elucidation of the modulating effects of compounds on colon cancer development has been limited and will be discussed further.

Dysplastic colonic crypts are recognized by their morphological changes in serial sections of the colon and have been considered to be early preneoplastic lesions of colon cancer (Chang, 1984; Deschner, 1974). Their limitation stems from the difficulties in their identification and enumerations which is time consuming and requires extensive knowledge of cytology pertaining to the colon. Such limitations circumvent their use as a means of quantifying the modulation of preneoplastic and neoplastic events by varying treatments (Bird, 1987).

Enzyme-altered foci have been identified in carcinogen treated rodent colonic crypts which otherwise exhibit normal morphological characteristics exhibit (Barrow et al., 1990; Pretlow et al., 1990, 1993). The foci are typified by the lack of hexosaminidase activity which is identified through specialized histological preparation and staining (Barrow et al., 1990). However, the role of hexosaminidase activity in the

carcinogenic process is not well established (Pretlow, 1994) and these focal lesions

have been identified in normal mucosa not exposed to carcinogen treatment (Barrow et

al., 1990). Incidentally few studies have utilized this system as a method to study

modulators of colon carcinogenesis which may stem from limitations such as time

consuming methods of analysis, unsubstantiated role of hexosaminidase in colon

carcinogenesis and lack of knowledge pertaining to their significance in normal colonic

mucosa.

Recently, the location of aggregates of lymphoid nodules (ALNs) have been

purported to play a promotional role in the *de novo* formation of adenocarcinoma

(Cameron et al., 1996; Carter et al.,1994; Hardman et al., 1991). However, there is a

lack of evidence supporting their role as preneoplastic colonic lesions and they have not

been assessed in the studies involving potential modulators of colon carcinogenesis. In

addition, ALNs are normal features of non-carcinogen treated rat colonic mucosa

(Cameron et al., 1996), which in itself does not lend support to their role as precursor

colonic neoplastic lesions.

A select few studies have reported the occurrence of other putative colonic

preneoplastic lesions utilizing different methods of microscopy (Barkla and Tutton,

1977; Cook et al., 1984; Kimura et al., 1984; Sandforth et al., 1988). Consequently,

these investigations did not further explore their significance in the etiology of colon

carcinogenesis beyond simple identification.

2.3.4.0 Aberrant crypt foci

Focal lesions of distinctly aberrant colonic crypt tissue architecture were

first identified in mice treated with a colon carcinogen (Bird, 1987). These were termed aberrant crypt foci (ACF) and proposed to be putative preneoplastic crypts. ACF have also been referred to as microadenomas (Corpet et al., 1990), precursor lesions (Rao et al., 1992) and dysplastic crypt foci (Caderni et al., 1991). It has been suggested that distinction among the terms should be employed with the terms microadenomas and dysplastic crypts restricted to those lesions that are confirmed histologically to display such histopathological features while reserving the term ACF for lesions identified topographically in intact non sectioned whole mucosa (Bird and Pretlow, 1992; Pretlow , 1994).

During the past nine years over one hundred studies on ACF have been published and have supported their role as preneoplastic lesions and their utilization in the study of rodent and human colonic neoplasia (Bird, 1995). The ACF model possesses several advantages over other precursor lesions in the study of colon carcinogenesis due to the simple and rapid method of identification and subsequent analysis. The identification of ACF does not require histological preparation or extensive knowledge of cytology (Bird et al, 1989), allows for the whole colon to be evaluated in a short period of time (Bird, 1987) and is achieved with low doses of carcinogen (McLellan and Bird, 1988a).

2.3.4.1 Biological properties of ACF

ACF are morphologically distinct from normal colonic crypts in that they are larger, have a thickened epithelial cell layer, exhibit a an elliptical shaped lumen and possess an increased pericryptal zone that separates them from the adjacent mucosa

(Bird, 1987, McLellan and Bird, 1988b). A plethora of studies have detailed the

biological properties of ACF. ACF have been; induced in mice and rats 2 weeks after

a single AOM injection (McLellan and Bird, 1988a); predominantly found in the distal

tumor appearing region of carcinogen-treated rodents colons (McLellan and Bird,

1988a; Pretlow et al., 1990); induced in a dose-dependent manner by colon carcinogens

(McLellan and Bird, 1988a; McLellan et al., 1991b; Tudek et al., 1989); observed to

display increased proliferative activity (McLellan et al., 1991b; Cheyer et al., 1993;

Otori et al., 1995); shown to exhibit varying degrees of nuclear atypia (McLellan et

al., 1991a); dysplasia (Pretlow et al., 1992a); shown to exhibit decreased

hexosaminidase activity (Pretlow et al., 1990); and demonstrated to display altered

mucin production (Caderni et al., 1995).

2.3.4.2 Genetic properties of ACF

Considering the growing evidence in support of a genetic model for colon

cancer development, the properties and potential aberrations of rodent ACF at the

genetic level have become of interest. ACF have been demonstrated to exhibit ;

mutations in the p53 gene (Stopera and Bird, 1993); elevated expression of c-*fos*

(Stopera et al, 1992); mutated K-*RAS* expression (Pretlow et al., 1995; Stopera et al.,

1992; Shivapurkar et al., 1994; Singh et al., 1994; Stopera et al., 1992; Tachino et al.,

1995; Vivonia et al., 1994; Zaidi et al., 1995); and DNA adduct formation (Qin et al.,

1994). Such studies lend support to the contention that ACF are preneoplastic lesions

which exhibit genetic alterations common to end stage tumors (Fearon and Vogelstein,

1990).

Figure 2.7 Topographic views (X40) of ACF consisting of various number of crypts in unsectioned methylene blue-stained F344 rat colon treated with 2 injections of 15 mg/kg body weight AOM. Figure 2.7 a.) depicts a single ACF surrounded by normal crypts. Note the increased size, thicker epithelial lining, larger pericryptal zone and eliptical lumen. Figures 2.7 b,c, and d depict ACF consisting of varying crypt multiplicities, 6, 7 and 10 respectively.

a b c d
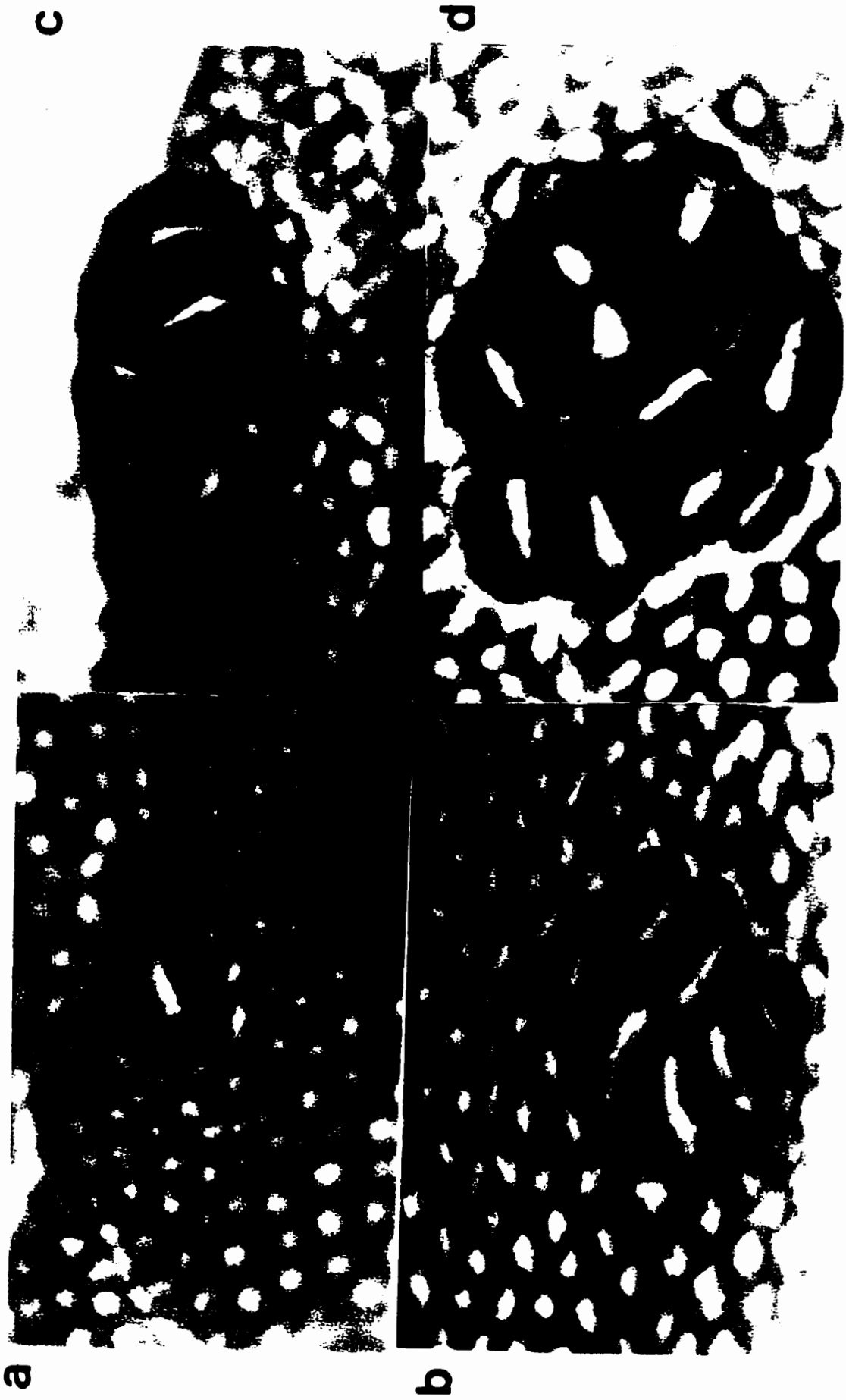
2.3.4.3 Biological heterogeneity among ACF

ACF exhibit a marked biological heterogeneity with respect to genotypic and phenotypic features (Bird, 1995). The heterogeneity in the topographical phenotypic features of ACF is illustrated in Figure 2.7. ACF may consist of one crypt (Figure 2.7 a) or several crypts (Figure 2.7b,c,d) with varying shapes and sizes. Such lesions may be identified within the same colonic mucosal surface. Sequential histologic analysis of ACF reveals that the number of ACF exhibiting dysplasia increases as well as the number of ACF with increasing number of crypts within a focus (crypt multiplicity) (Bird, 1995). The presence of dysplasia also appears to be quite variable from one ACF to another (Roncucci et al., 1991a) suggesting that dysplasia and clonal expansion of ACF may be regulated by separate biological events (Bird, 1995). Consequently, heterogeneity of ACF is in agreement with heterogeneity identified in other preneoplastic colonic lesions. Pretlow and colleagues (1994) have demonstrated heterogeneity in enzyme altered foci and Mayer and colleagues (1987) have identified heterogeneity among mucus secreting preneoplastic crypts.

2.3.4.4 ACF in human colonic mucosa

A growing number of investigations are identifying ACF in human colonic mucosa with striking similarities to lesions identified in rodent models. Pretlow and colleagues (Pretlow et al., 1991, 1992) and Roncucci and colleagues (1991, 1992) independently reported the presence of ACF-like lesions in human colonic mucosa associated with high risk for developing cancer. The structural similarities between human and rodent ACF have been established are reviewed (Pretlow, 1994). Emerging

research in human colon is directed towards determining both the phenotypic and genotypic atypia in ACF in relation to normal and neoplastic tissues. Human ACF demonstrate: altered enzyme activity (Pretlow, 1994); hyperplasia (Otori et al., 1995); K-*RAS* mutations (Yamashita et al., 1994); somatic APC mutations (Smith et al, 1994); genomic instability (Augenlicht et al., 1996); expression of carcinoembryonic antigen (Pretlow et al., 1994). Such investigations provide impetus for the use of ACF in animals as a model for studying human colon cancer (Pretlow, 1994).

2.3.4.5 ACF as a bioassay for colon carcinogenesis

The preceeding discussions have substantiated the role of ACF as precursor lesions in colonic cancer in human and animal models, thus the use of this system in determining the effects of potential modulators of the disease process is validated (Bird, 1995). Firstly, as ACF are induced by known colon carcinogens (McLellan and Bird, 1988a; Bruce, 1990; Takahashi et al., 1991; Tudek et al., 1989) and, therefore, may be used to further identify potential colon carcinogens. Secondly, ACF may be used to assess the effectiveness of various chemopreventive agents involved in the prevention of tumorigenesis (Kawamori et al., 1995; Mereto et al., 1994; Reddy et al., 1994; Wargovich et al., 1992, 1996;) and of agents known to promote tumorigenesis (Corpet et al., 1993; Bird, 1995; Pereira et al., 1994; Sutherland and Bird, 1994; Zarkovic et al., 1993). Lastly, relevant to the present dissertation, the ACF model may be used as a bioassay to explore the role of diet in cancer etiology (Alabaster et al., 1995; Bird et al., 1996; Bird and LaFave, 1995; Bruce et al., 1993; Caderni et al., 1991; Corpet et al., 1995; Kendall et al., 1992; Kilkarni and Reddy, 1994; Kawamori et al., 1994,

1995; Kristiansen et al., 1995; LaFave et al., 1994; McLellan and Bird, 1991; Rao et al., 1993; Shivapurkar et al., 1992; Stamp et al., 1993; Takahashi et al., 1993, 1994; Thorup et al., 1994; Tudek et al., 1989; Pretlow et al., 1992b; Yao et al., 1996; Young et al., 1996; Zhang et al., 1993).

Across all three categories of investigation, several growth characteristics of ACF development are determined in order to assess the progress of the disease and the effectiveness of the agent, or diet, in their modulation. Modulation studies are initially induced by the chemical carcinogens, the animals are allocated into control and treatment groups, and the growth characteristics of ACF are compared after a varying time point as evidence of the promotion or inhibition of colon cancer by the treatment. There are three main characteristics of ACF that can be quantified which include; the total number of ACF per colon; the size (area) of individual ACF; and the crypt multiplicity or the number of crypts within the focus (McLellan and Bird, 1988b). The total number of ACF is the most common parameter assessed throughout the ACF literature. However, it has been demonstrated that crypt multiplicity may be the characteristic which best predicts colonic tumor incidence in rat (Magnuson et al., 1993; Zhang et al., 1992).

Incidentally, the response of ACF growth to tumor promoters, inhibitors and diet have been variable (Caderni et al., 1991; Bird, 1996; Corpet et al., 1990; Pareira and Khoury, 1991; Rao et al., 1992). This becomes very evident when rats were fed diets containing cholic acid (a known tumor promoter) which appeared to inhibit the total number of ACF in the colon (Magnuson and Bird, 1993; Magnuson et al., 1993;

Bird, 1995). However, cholic acid appeared to eliminate those lesions of a lower crypt multiplicity and enhance the growth of those with a higher multiplicity (Magnuson et al., 1993). Consequently, discontinuing the cholic acid diet resulted in a rapid increase in the number of ACF, establishing that the growth inhibitory effect of the cholic acid diet was reversible (Bird, 1995). This cholic acid model provides evidence that biological differences exist among ACF of varying crypt multiplicities (Bird, 1995) and may permit the detection of those lesions which have attained a certain level of autonomy, enabling them to successfully transduce into tumors (Bird et al., 1996). This model also supports the contention that the disease process may be regressed or retarded as a result of intervention.

Such evidence may provide some explanation towards the variability among investigations employing the ACF bioassay. The emerging conclusions may be dependent on the growth characteristic assessed and the nature of the experimental protocol. In order to delineate some of these effects, sequential and comprehensive analysis of growth characteristics may be necessary.

2.3.5 Dietary fat, ER , age, and colon carcinogenesis

The interest in disease prevention as a means for reducing the costs of health care is a paramount issue in present day society. As previously mentioned the link between cancer and dietary fat is evident and has lead to several dietary recommendations by such agencies as Health Canada. Such recommendations are reflected in the Canada food guide which recommends increased consumption of fruits and vegetables, decreased intake of total fat, in particular saturated fat. In addition, the

fastest growing population in society is the elderly. Thus the health status of these individuals directly affects health care costs. A plethora of investigations have explored the effects of diet on the aging process. Incidentally, the incidence of cancer is greater among the elderly (Weindruch et al., 1991). Such evidence substantiates the importance of utilizing animal models to study the effects dietary fat, ER, and age on colon carcinogenesis in order to further understand the mechanisms of the disease and its ability to be modulated by changes in diet composition.

2.3.5.0 Dietary Fat and Colon Carcinogenesis

Several studies have demonstrated that increasing the amount of fat (5% increased to 20% w/w) in rodent diets serves a promotional effect on chemically induced tumor development (Kumar et al., 1990; Kritchevsky, 1993; Rao et al., 1993; Reddy, 1986). However, the promotional effect is effective for corn oil, safflower oil, beef fat, and lard (Carroll, 1991; Nigro et al., 1975; Rao et al., 1991; Reddy et al., 1984) yet sources of dietary fat derived from fish (Minoura et al., 1988; Narisawa et al., 1998; Reddy and Maruyama, 1986; Reddy and Sugie, 1988; Reddy et al., 1991) and coconut (Reddy and Maruyama, 1986) appear to inhibit colon carcinogenesis.

It has been proposed that the promotional effect of a high fat diet may stem from the increased amount of bile acid released in the intestine due to the increased fat content (Bruce and Bird, 1986). Bile acids have been demonstrated to act as promoters of experimental colon carcinogenesis (Bird, 1995; Bruce and Bird, 1986; Sutherland and Bird, 1993; Magnuson et al., 1993). The tumor inhibitory and promotional effect of fats from different sources stems from the hypothesis that

alterations in dietary fat may exert their effects by altering the colonic membrane structure and function (Fischer et al., 1981; Hopkins and West, 1976). Fish oil is rich in highly polyunsaturated ω3 fatty acids such as eicosapentanoic and docosahexanoic acid (Takahashi et al., 1993) while corn oil is rich in linoleic acid (Carroll et al., 1986). Prostanoids derived from membrane lipids may be important in the tumorigenic process (Bennet, 1981;Farm et al., 1981; Jaffe, 1974). It has been hypothesized that the increased linoleic acid content of corn oil serves as a precursor to prostaglandin synthesis which promotes tumorigenesis (Carroll et al., 1986). Conversely the docosahexanoic content of fish oil has been shown to be a strong inhibitor of prostaglandin synthesis (Corey et al, 1983). The enzymes involved in the metabolism of phospholipid derived prostaglandins have also been shown to be affected by the amount and type of dietary fat (Rao et al., 1996, Rao and Reddy, 1993).

Feeding different types of fat has been demonstrated to effect phospholipid composition of normal mucosa (Robblee et al., 1988). It has been demonstrated that the preneoplastic colon epithelium differs from that of normal epithelium with respect to prostanoid synthesis (Robblee and Bird, 1994). Increased dietary fat has been demonstrated to enhance the development of ACF (LaFave et al., 1994; McLellan and Bird, 1990; Shivapurkar et al., 1992). In addition, the type of fat affects the development of ACF (Bird and LaFave, 1995; Shivapurkar et al., 1992; Takahashi et al., 1993, 1996;). Generally, the diet containing fish oil or its derivatives resulted in decreased crypt multiplicity and total number of ACF (Takahashi et al, 1993, 1996). Corn oil exerted a growth enhancing effect on ACF with a higher crypt multiplicity in

relation to olive oil, however this effect was dependent on the dose of AOM (Bird and LaFave , 1995). Dietary cholesterol has also been demonstrated to enhance the total number of ACF in murine colons (Kendall et al, 1992).

The stage or stages at which dietary fat exerts its most prominent effects has not been established. The majority of investigations feeding either a low or high fat diet immediately after carcinogen treatment and continue until end stage tumor development. A recent study by Bird and colleagues (1996) established that feeding a high fat diet during the early stages of colon carcinogenesis provided a tumor enhancing effect that was not mitigated by the reduction of dietary fat in the later stages of carcinogenesis. This study provides evidence that the early stages of colon carcinogenesis are more susceptible to changes in diet.

2.3.5.1 ER and colon carcinogenesis

The concept of a relationship between ER and tumorigenesis began in the early 1940s when Tannenbaum and colleagues (Tannenbaum, 1945a, 1945b, Tannenbaum and Silverstone, 1953) indicated that ER inhibited spontaneous and chemically-induced mammary and skin tumors. Interest in ER as a potent inhibitor of tumorigenesis waned in the ensuing decades followed by a resurgence of interest in the mid 1980s. The first study to demonstrate that ER was a potent modulator of chemically induced colonic tumors was conducted by Pollard and Luckert (1985) who demonstrated that the effects of ER were dependent on the type of carcinogen. Kritchevsky et al., (1986) demonstrated that ability of 40%ER to significantly inhibit the development of DMH-

induced colon tumors. Reddy and colleagues (1987, 1991) have demonstrated the modulating ability of ER in colonic mucosa treated with the carcinogen AOM. In addition, they explored the effects of different levels of ER on colonic tumor incidence and demonstrated that a level of 20% ER (in high fat diets) or greater was necessary to inhibit the development of tumors (Kumar et al., 1990).

However, the amount of fat in the diet has confounded the issue of ER in colon tumorigenesis. Significant inhibition of colonic tumors by 40% ER has been observed even though the fat content of the diet was more than double that of the *ad libitum* group (Klurfeld et al., 1987). Similar findings have been reported in mammary carcinogenesis (Boissonneault et al., 1986). Thus it has been hypothesized that ER is a " more stringent determinant of tumor growth than fat intake" (Kritchevsky et al., 1986). ER studies conducted by Reddy and colleagues (1991, 1987) remove energy from the ER diets from carbohydrate sources only while keeping the amount of fat (w/w) equal in relation to the groups eating freely. Therefore the interest in comparing the effects of energy from fat with the effects of energy from carbohydrate in conjunction with ER emerged. Birt and colleagues (1993) addressed this issue in an investigation involving chemically induced skin tumorigenesis. Restriction of energy from either fat or carbohydrate delayed the rate and reduced the incidence of carcinoma development (Birt, 1993). Incidentally, the issue of energy from fat and carbohydrate remain unresolved (Kritchevsky, 1993).

The effect of ER on enzymes purported to play a role in colon carcinogenesis have been limited to one study in which 20% ER significantly decreased ornithine

decarboxylase and tyrosine kinase activity in normal mucosa and tumors (Kumar et al., 1990).

Similar to the findings of dietary fat (Bird et al., 1996) it has been proposed that the earlier the ER is begun, the greater the inhibition of tumor growth (Kritchevsky, 1993). However, the effects of ER on the early versus the later stages of colon carcinogenesis have not been investigated.

The mechanisms by which ER modulates colon carcinogenesis have not been elucidated. Considering the vast physiological effects of ER, described previously, several mechanisms involving such things as circulating growth factors, enhanced DNA excision and repair, cell-mediated immunity, less energy available for cell proliferation, and oncogene expression to name a few may be involved in colon carcinogenesis. At the present time, these parameters have not been measured in colon carcinogenesis in conjunction with ER.

2.3.5.2 Age and colon carcinogenesis

Chemically induced colonic tumors occur in older rats which is more of a reflection of the time required for the lesion to develop (Weindruch, 1992). However, the relationship between modulation of age associated cellular changes and colonic tumor development have not been addressed in the scientific literature. Despite the growing evidence that changes in diet such as ER are able to affect age associated changes in parameters associated with cancer development (Leakey et al., 1992, Sachan, 1982), this area of research remains dormant. Majumdar and colleagues

(1988, 1989, 1990, 1992) have demonstrated age associated changes in tyrosine kinase activity and EGF expression in carcinogen treated and non-carcinogen treated colonic mucosa. These parameters have been suggested to play a role in colon carcinogenesis (Kumar et al., 1990). Such studies provide the impetus towards exploring the effects of factors which modulate age associated changes involved in colon carcinogenesis.

2.4 Summary

A culmination of the studies reviewed in this dissertation provided the stepping stones upon which the hypotheses began to evolve. The ACF system, as a bioassay, is a valuable tool for the study of colon carcinogenesis. Its usage allows for the sequential analysis of the varying stages of colon carcinogenesis preceeding the appearance of tumors. Therefore, ACF may be employed to study the effects of potential modulators of the disease process, such as fat, and ER. Potential mechanisms involved in the inhibitory effects of ER and promotional effects of fat on colon tumor development are not well established. Growth characteristics of ACF, and enzymatic parameters, as affected by such dietary variables may lend insight towards the preneoplastic state of the colon as well as the mechanism(s) by which they modulate the disease. In addition, the response of ACF growth to changes in fat and ER in young and old rats may provide further knowledge towards understanding age associated changes in colon cancer incidence. The stage at which diet intervention is paramount has not been established. Therefore, altering the amount of fat and energy in the diet at varying stages may also further elucidate the susceptibilities of

preneoplastic lesions to modulation by diet.

Each study undertaken in this dissertation has stemmed from one or more than one of the above mentioned hypotheses. The individual research goals and specific objectives of each study are detailed at the beginning of each chapter.

3. MATERIALS AND METHODS

Methods used in more than one study are summarized in this chapter.

Most chemicals were purchased from Sigma Chemical Co., Mississauga, Ontario,

Canada unless specified otherwise.

Quantification of ACF

All rats were killed by CO_2 asphyxiation. Colons were removed, flushed with

cold phosphate buffered saline (PBS), slit longitudinally from caecum to anus and fixed

flat on filter paper in either 10% neutral buffered formalin or 70% ethanol. After a

minimum of 24 hours fixation, colons were stained for 15-20 minutes in 0.2%

methylene blue dissolved in PBS. ACF were identified and quantified by light

microscopy. The colons were viewed mucosal side up at a magnification of 10X.

Characteristics of ACF used to distinguish them from normal crypts have been

previously described (McLellan et al., 1991). The number and multiplicity of ACF

were determined along the entire length of the colon. Crypt multiplicity refers to the

number of crypts per focus and was analysed in several ways. Average crypt

multiplicity represents that average of the mean number of crypts in a focus per colon

or per group. In addition, ACF with different growth features were further grouped

based on their crypt multiplicity into small (1-3 crypts/focus), medium (4-6

crypt/focus) and large ACF (> 6 crypts per focus). These classifications varied slightly

from one study to another which is detailed in their individual material and methods

sections.

Cell proliferation

Mitotic figures for the metaphase arrest technique is described in chapter 7.

Proliferating cell nuclear antigen expression (PCNA) was determined employing

immunohistochemical techniques based on the method of Richter and colleagues

(1992). Approximately 1-cm^2 proximal and distal sections of the colons fixed in 70%

ethanol were used. The tissues were embedded in paraffin wax and sectioned at a

thickness of 5-μm. Longitudinal unstained colonic crypt sections were mounted onto

slides. Immunohistochemical techniques were carried out utilizing the bulk Histo-Stain

SP kit from Zymed (London, Ontario, Canada). Tissue sections were deparaffinized

and flooded with normal goat serum and incubated for 20 minutes to block nonspecific

binding. Anti-PCNA monoclonal antibody (Dimension Laboratories, Inc. Mississauga,

Ontario, Canada) was diluted in PBS (1:40), applied to the sections and incubated for 1

hour. The sections were then incubated with the anti-mouse IgG (antibody bridge) for

20 min followed by incubation with mouse IgG peroxidase (labelling agent) for 20 min.

The peroxidase reaction was then initiated by immersing the slide in 0.06% 3,3'-

diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 for 10-15 min. The slides were

counterstained with haematoxylin (Sigma), dehydrated and mounted with Permount

(Fisher Scientific, Ottawa, Ontario, Canada). Incubations through the procedure were

carried out in a humid ambient chamber at room temperature, and were washed

extensively with fresh PBS in between incubations. PCNA labelled cells were

determined in ten crypts per colon and classified as darkly stained cells along the length

of the crypt. The number and position of the labelled cells in each crypt were

recorded. The proliferative zone was defined as the highest labelled cell position along

the length of the crypt, with the base of the crypt as the bottom and the mouth of the crypt as the top. PCNA labelling index (LI) was calculated as the number of labelled cells per crypt divided by the total number of cells along both sides of the crypt and multiplied by 100.

Extraction, Separation of Colonic Cytosol and Membrane Fractions

Fresh and thawed mucosa (formerly frozen at -80° C) 0.3 grams, from all groups were placed in ice cold cytosolic buffer (3 grams mucosa in 3 ml buffer) (pH 7.5) containing 25mM tris, 0.25mM PMSF (phenylmethylsulfonyl fluoride) 5mM EGTA, 5mM EDTA, 15mM mercaptoethanol, 0.25M sucrose, 10 μg/ml leupeptin, pepstatin 10 μg/ml, 40 μg/ml aprotinin and 10μg/ml trypsin inhibitor. All tissues were then homogenized with a polytron and centrifuged at 33,000 rpm (100,000 x g) rpm for 1 hour. The supernatant drawn was the cytosolic fraction and was placed on ice. The pellet was removed and placed in 3 mls of ice-cold membraneous buffer, which is the cytosolic buffer with the addition of Triton X-100 (0.8% v/v), and homogenized briefly and centrifuged at 33,000 rpm (100,00 x g) for 1/2 hour. The resulting supernatant drawn was the membraneous fraction and was placed on ice until the time of the assay.

PKC Assay

Colonic PKC activity was measured utilizing a Biotrak kit from Amersham Life Sciences (Arlington Heights, IL). The kit measures PKC activity based upon the PKC catalysed transfer of the γ-phosphate group of adenosine-5'-triphosphate to a peptide which is specific for PKC. The reaction mix provided in the kit included four buffers,

specifically; 1)lipid (0.3mg/ml Lα-phosphatidyl-L-serine, 24 μg/ml phorbel 12-

myristate 13-acetate); 2.)specific PKC peptide buffer (900 μM peptide in 50mM); 3.)

calcium buffer (12mM calcium acetate); 4.) DTT buffer (30mM dithiothreitol). All

four buffers were in 50mM Tris/HCl 0.05% (w/v) sodium azide, pH 7.5. Radio

labelled γ-P^{32} ATP (NEN, Boston, MA) with a specific activity of 3000Ci/mmol was

added to the kit magnesium ATP buffer (1.2mM ATP in 30mM Hepes, 72mM MgCl$_2$)

at a level of 0.5μCi/5μl of buffer. Preliminary studies conducted to optimize this

method revealed a linear response for approximately 50-100 μg of protein from the

cytosolic and membranous fractions. Therefore, this amount was utilized in each

assay. Initially, the buffers 1-4 were thawed on ice and mixed in equal volumes. Each

assay required 25 μl of this buffer reaction mixture. Cytosolic and membrane fractions

were placed in 600 μl eppendorf tubes, and briefly mixed with 25 μl of the buffer

reaction mix. The assay was started by the addition of 5μl of the MgCl$_2$ γ P^{32} ATP

buffer and allowed to incubate for 15 minutes at 37°C. The reaction was terminated

with 10 μl of the kit stop reagent (300mM orthophosphoric acid with carmosine red).

The complete assay mixture was then spotted onto peptide binding paper provided by

the kit and allowed to dry. The binding papers were then subjected to three washes

with 10% acetic acid for 10 minutes each. Acetic acid was then decanted and the

binding papers were transferred into scintillation vials containing 10 ml of Cyto-Scint

(ICN, Montreal, Canada) and counted for 1 min with an LS-6000TA Beckman

Scintillation Counter. Duplicates of each sample were performed. Each colonic

fraction was assayed in the presence of the sample (colonic fraction), ATP and reaction

mix (buffers 1-4). In order to account for endogenous phosphorylation the activity was

assayed in the presence of the sample, ATP and reaction mix without the peptide buffer

(buffers 1,3,4). In addition, the exogenous phosphorylation was determined by

assaying the cytosolic and membranous buffers with the reaction mix and ATP buffers.

Specific activity was expressed as pmol Pi transferred/min/mg protein.

Sample Calculation of PKC activity (V):

1. Calculation of specific activity of ATP (SA)

5 μl of ATP mix contains 6×10^{-9}M ATP

$$SA = \frac{dpm\ per\ 5\ \mu l\ ATP\ mix}{6}\ cpm/nmol$$

2. Calculation of activity of sample minus blank (S)

Activity of (reaction mix (buffer 1-4) + cell fraction + ATP) - Activity of (reaction
mix (buffer 1,3,4)+ cell fraction +ATP) = S

3. Calculation of pmol ATP transferred per minute per mg protein (V)

$$V = \frac{S}{(SA)(15min)(mg\ protein)}$$

This method of calculation was also applied to the Tyrosine Kinase (TK) and

Epidermal Growth Factor Receptor Tyrosine Kinase assays, with slight modifications

detailed in Chapters 4 and 9.

Protein Analysis

Concentration of protein was determined according to Bradford (1976) using a

Coomassie protein assay reagent (Pierce, Rockford, Il.). Bovine serum albumin was

used as the standard.

Detection of Antigens by Western Blotting

Cytosolic and membrane fractions were extracted and protein concentration

determined according to the protocols stated previously. The buffer systems and gel

recipes for western blotting techniques are detailed in appendix A. Twelve μg of

protein from each sample was combined and boiled (90°C for 1 minute) with an equal

volume of 2X concentration SDS (sodium dodecyl sulphate) sample buffer. Samples

were separated on a discontinuous gel denaturing gels system using 0.75mm 5%

stacking and 10% separating SDS-PAGE (polyacrylamide gels) as per the method of

Laemmli, (1970) in 600ml running buffer at 180 volts for 1 hour. The gels were then

transferred to nitrocellulose transfer membranes (Amersham) utilizing a BIO-RAD

(Mississauga, Ontario) Mini-Protean cell in approximately 600 ml transfer buffer at

100 volts for 2 ½ hours. To ensure equal loads and even transfer, the gels were

stained with coomassie blue (appendix A). Immunoblotting was carried out according

to the suggested protocol by Transduction Laboratories (Lexington, Kentucky). The

membranes were blocked for 1 hour, with rocking, with 5% nonfat dry milk powder in

TBS-T (Tris buffered saline with 0.1% Tween-20), then washed for 30 min (5x 50 ml)

with TBS-T. Membranes were then probed with the primary monoclonal anti- PKCα,

γ, δ, ϵ, ζ mouse IgG (Transduction Laboratories, Lexington, Kentucky) diluted 1:1000

in 5% skim milk powder TBS-T overnight at 4°C. For each antibody, a particular

fragment of the specific PKC isoform, corresponding to particular amino acid residue

positions, were used as immunogens. Cross reactivity is reported between PKC α and γ

only. After incubation with the primary antibody, the membranes were washed for 30

min (5x 50 ml) with TBS-T. Incubation with the secondary antibody, rabbit anti-

mouse horse radish peroxidase (HRP) conjugated IgG (Sigma Chemical Co.), was done

at a dilution of 1:1000 in 5% skim milk powder in TBST-T for 1 hour at room

temperature. Membranes were subjected to a final wash with TBST-T and

immunoreactivity was detected using an enhanced chemiluminescence (ECL) kit

(Amersham, Arlington Heights, IL) and Kodak x-OMATAR film. To insure that the

detection of immune-complexes by ECL were linear initial trials were conducted utilizing

increasing amounts of protein. The response was linear between 2 and 25 μg of protein.

The resulting films were scanned using a Reliasys Image Scanner and Photoshop version

2.5 software for Macintosh. The area of the protein bands (mm^2) detected on the film

was then calculated using Image version 1.49 software. This method of quantification

involved an error of approximately ±0.05 mm^2. Areas calculated were then normalized

with the largest area normalized to one and lesser values scaled proportionally. This

allowed for comparison of expression from one film to another, but within the same

antibody. Statistical analysis of protein band areas were limited to comparisons of the

same antibody.

4. COLONIC PROTEIN KINASE C (PKC) AND TYROSINE KINASE ACTIVITY IN MALE F344 RATS AS AFFECTED BY DIETARY FAT, ENERGY RESTRICTION (ER) AND AGE

4.1 Introduction

Several investigations have provided evidence that the PKC isozymes, members of serine/threonine kinases, are involved in the regulation of cell growth, differentiation, apoptosis and many other cellular functions (de Vente et al., 1995; Dlugosz and Yuspa, 1993; Nakamura and Nishizuka, 1994; Nishizuka, 1992). In addition, PKC has been implicated to play a role in a plethora of gastrointestinal functions including pancreatic (Merrit and Rubin, 1985) and gastric secretions (Anderson and Hanson, 1985) and malignant colonic transformation (Guillem et al., 1987; Kopp et al., 1991; Kusunoki et al., 1992; McGarrity and Peiffer, 1994). Enhanced PKC activity has been associated with increased cell proliferation, and therefore, increased risk of neoplastic development, whereas decreased activation has been suggested to decrease such a risk. Colonic PKC activity may have been shown to be significantly altered by carcinogen treatment (Craven and DeRubertis, 1992b), exposure to bile acids (Craven et al., 1987) and dietary fat (Lafave et al., 1994). Restricting the level of available dietary energy (kcal intakes) has also been shown to significantly modify PKC activity in epidermal skin cells (Kris, et al., 1994) and in non-carcinogen treated colonic mucosa subjected to a 48-hr fast (Craven and DeRubertis, 1992a).

The distribution of PKC isozyme expression in human and rat colon, utilizing immunocytochemical and immunoblotting procedures, has been the focus of several recent investigations (Davidson et al., 1994,1995; Jiang et al., 1995; and McGarrity et al., 1996). PKC α ,β, δ,ϵ, and ζ have all been identified as isoforms expressed in rat and human colonic mucosa. It has been proposed that the individual isozymes may have specialized and distinct functions in cell signalling due to their differences in cofactor requirements and substrate specificity (Hug and Sare, 1993). Consequently, dietary fat and fiber have both been shown to alter colonic PKC isozyme expression in rat colons.

Another family of enzymes involved in cellular phosphorylation reactions is the protein tyrosine kinases (TK) (Hunter and Cooper, 1985). TK activity appears to be elevated in proliferating normal and neoplastic cells (Hunter and Cooper, 1985). Therefore, lowering TK activity has been suggested to be a preventive measure in neoplastic cells (Gazit et al., 1989). Elevated levels of TK activity have been demonstrated in the carcinogen treated rat colon (Arlow et al., 1989; Rao et al., 1993; Singh et al, 1992). In addition, elevated EGF-R TK (epidermal growth factor receptor tyrosine kinase) has been shown to be activated by carcinogen treatment in rats (Malecka-Panas et al., 1996) and in human colon carcinoma cells (Radinsky et al., 1993). Two TKs, c-YES in a human colon carcinoma cell line (Park et al., 1993) and c-SRC in premalignant colonic epithelia of ulcerative colitis patients has been demonstrated to be elevated in relation to controls. Changes in diet composition have been associated with decreased levels of TK activity specifically by including dietary curcumin (a phenolic compound) (Rao et al., 1993) and calcium (Arlow et al., 1989). Changing the amount of available

energy in the diet has long been associated with a decreased tumor incidence in several

tissues including chemically induced colonic tumors (Klurfeld et al., 1987; Kumar et al.,

1990; Reddy et al., 1987). Furthermore, energy restriction delays a variety of age-

associated pathological conditions and may retard deterioration of a plethora of

physiological process associated with aging in rodents (Holehan and Merry, 1986;

Masoro, 1985).

The evidence provided in the previously cited research provided the impetus for

the present study in which the effect dietary fat and energy restriction, as related to the age

of the rat, on colonic PKC and TK activity was explored.

The objectives of this study were as follows; a) to determine the effects of age on

colonic PKC and TK activity; b) to determine the effects of varying levels of fat and

energy on colonic PKC and TK activity; and c) to identify PKC isozyme expression in

cytosolic and membraneous fractions of colonic mucosa.

4.2   Material and Methods

Animal Care and Diets : The animals were housed in wire cages with sawdust bedding

with a 12h light-dark cycle. Temperature and humidity were controlled at 22°C and 55%

respectively. Animals were given laboratory chow and water ad libitum until initiation of

the experiment. All animals were cared for according to the guidelines of the Canadian

Council on Animal care. The diets were based on a semi-synthetic AIN-76A (American

Institute of Nutrition, 1977;1980) standard diet with modifications specified in Table 4.1.

Table 4.1
Composition of HF(high fat), HFER (high fat energy restricted), LF (low fat), LFER (low fat energy restricted) diets.

COMPONENT		%COMPOSITION		
	HF	HFER	LF	LFER
		g/100g		
Casein	23.00	23.00	20.00	20.00
D-L Methionine	0.30	0.30	0.30	0.30
Corn Starch	33.75	31.46	52.00	50.06
Dextrose	8.52	8.52	13.00	13.00
Corn oil	23.00	23.00	5.00	5.00
Cellufil	5.90	7.08	5.00	6.00
AIN-76 Min.* Mix.	4.11	4.93	3.50	4.20
AIN-76 Vit.* Mix.	1.18	1.42	1.00	1.20
Choline Bitarate	0.24	0.29	0.20	0.24

*Refer to appendix D for content.

Study Design:

40 weanling and 40 10 week old male F344 rats (Charles Rivers, Montreal Canada) were used. After one week of acclimatization on standard lab chow, the animals were housed in single cages and randomly allocated 10 per group into 8 experimental groups. These groups were fed one of the HF, HFER, LF or LFER within the young and old groups . Diet constituents were purchased from Amersham (Arlington Heights, IL) with the exception of corn oil (Mazola Brand, Canada Safeway). The diets and the feeding regime were based on those described by Kumar and colleagues (1990). Energy was removed from the HFER and LFER diets from corn starch alone. These diets were also supplemented with increased levels of cellufil, vitamins, and minerals to ensure equal intake of these constituents between the ER and their ad libitum counterparts. Diets within each fat classification were relatively isocaloric, therefore ER (20%) was accomplished by feeding the ER groups 80% of the average daily intake of their respective HF and LF ad libitum counterparts. Initial and weekly body weights were recorded. After termination (12 weeks of feeding), the colons were removed, flushed with phosphate buffered saline (PBS), slit from the caecum to the anus and placed on a cooled surface at 4°C and mucosal scrapings were collected. One half of the scrapings (5/group) were placed immediately into the cytosolic buffer for the PKC assay. The other half (5/group) were designated for the TK assay and placed into sterile cryo-vials, frozen immediately in liquid nitrogen and stored at -80° C for approximately 2 months.

Extraction, Separation of Colonic Cytosol and Membrane Fractions

Colonic cell fractions were separated and prepared according to the method in

Chapter 3.

PKC Assay

Colonic PKC activity was measured utilizing a Biotrak kit from Amersham Life Sciences and described in Chapter 3. Activity was expressed as pmol Pi transferred/min/mg protein.

TK assay

TK activity was measured utilizing a method developed by Rao et al., 1993. Approximately 20-50 μg of membrane fraction protein was incubated in a 50 μl reaction mix containing 50mM Tris (pH 7.5), 20mM $MgCl_2$, 0.02% Triton X-100, 50μM Na_3VO_4, 20μg/50μl of the glutamic acid-tyrosine polymer (4:1), and 50 μm ATP. The reaction was started by the addition of radio labelled γ-P^{32} ATP (NEN, Boston, MA) with a specific activity of 3000Ci/mmol and added at a level of 0.5μCi per assay tube. The assay was incubated for 15 min at 24°C and stopped by spotting on Whatmann #3 filter paper. The filter papers were allowed to dry and subjected to 3 washes with 10% trichloroacetic acid (TCA) with 10 mM sodium pyrophosphate. Following the washes, the filter papers were placed in 10 ml scintillation vials with 10 ml Cyto-Scint (ICN, Montreal, Quebec, Canada) and counted for 1 min with an LS-6000TA Beckmann Scintillation counter. TK activity was calculated as the difference in activity assayed in the presence of the sample (colonic fraction), ATP and reaction mix and the activity assayed in the presence of the sample, ATP and reaction mix without the glutamic acid-tyrosine polymer. Specific activity was expressed as pmol

Pi transferred/min/mg protein.

Protein Analysis

Protein analysis was determined using the method developed by Bradford (1976) and detailed in Chapter 3.

Detection of Antigens by Western Blotting

The detection of PKC α and γ isoform in young and old rat cytosolic and membranous fractions were carried out according the methods detailed in Chapter 3.

Statistical Analysis

Statistical analysis of the data was carried out using Analysis of Variance (ANOVA) in conjunction with Duncan's Multiple Range Test using the SAS statistical software package for microcomputers (SAS Institute Inc., Cary, NC). The two-way ANOVA was also used to determine the main effects of fat, ER and possible fatxER interaction. A P value ≤ 0.05 was considered significant.

4.3 Results

Body weight and food consumption

Initial body weights for the old (10 week) rats were significantly higher than the initial body weights for the weanling rats (Figures 4.1 and 4.2). As early as the second week of feeding the diets, significant changes in body weight were observed. In general, weights of the ER were lower than their HF and LF counterparts from the second week of feeding until the 12th week among the old and young rats . In relation to the HF and LF groups, by week 6 the HF weights were greater than the LF group in the old animals (Figure 4.2), however within the young animals the weights of the HF and LF did not

differ over the duration of the experiment (Figure 4.1).

Colonic PKC activity

With the exception of the older LFER group, membrane colonic PKC activity was higher in all groups, in both age categories, as compared to the cytosolic activity (Table 4.2). There was no significant diet effect on PKC activity in either fraction. The high variance from one animal to another within a group, resulting in large standard error of the mean, may account for this observation. Variance within the older groups appeared to be less than in the younger groups. However, there was a trend for the membranous and cytosolic PKC activity to be higher in the high fat (HF and HFER) and the ad libitum groups within each age category. Membrane associated PKC activity was significantly affected by the age of the rat (two-way ANOVA, $P = 0.004$) (Table 4.3) with the younger animals exhibiting higher activity. The statistical analysis of percentage of cytosolic and membranous PKC activity, relative to the total activity, did not reveal any significant diet or age effects (data not shown). The measurement of PKC in non-injected colonic mucosa utilizing this particular method has not been investigated previously, making it difficult to speculate about the ranges of calculated values for PKC activity (expressed as pmol Pi/min/mg protein) observed in this study. The statistical analysis of percentage of cytosolic and membranous PKC activity, relative to the total activity, did not reveal any significant diet or age effects (data not shown).

Figure 4.1 Average body weights (values are mean±SEM[bars]) of young F344 rats fed

one of four diets, HF, HFER, LF, LFER for 12 weeks.   Means not sharing a common

superscript (^{a,b}) are significantly different (P ≤0.05, repeated measures ANOVA and

Duncan's multiple range test).
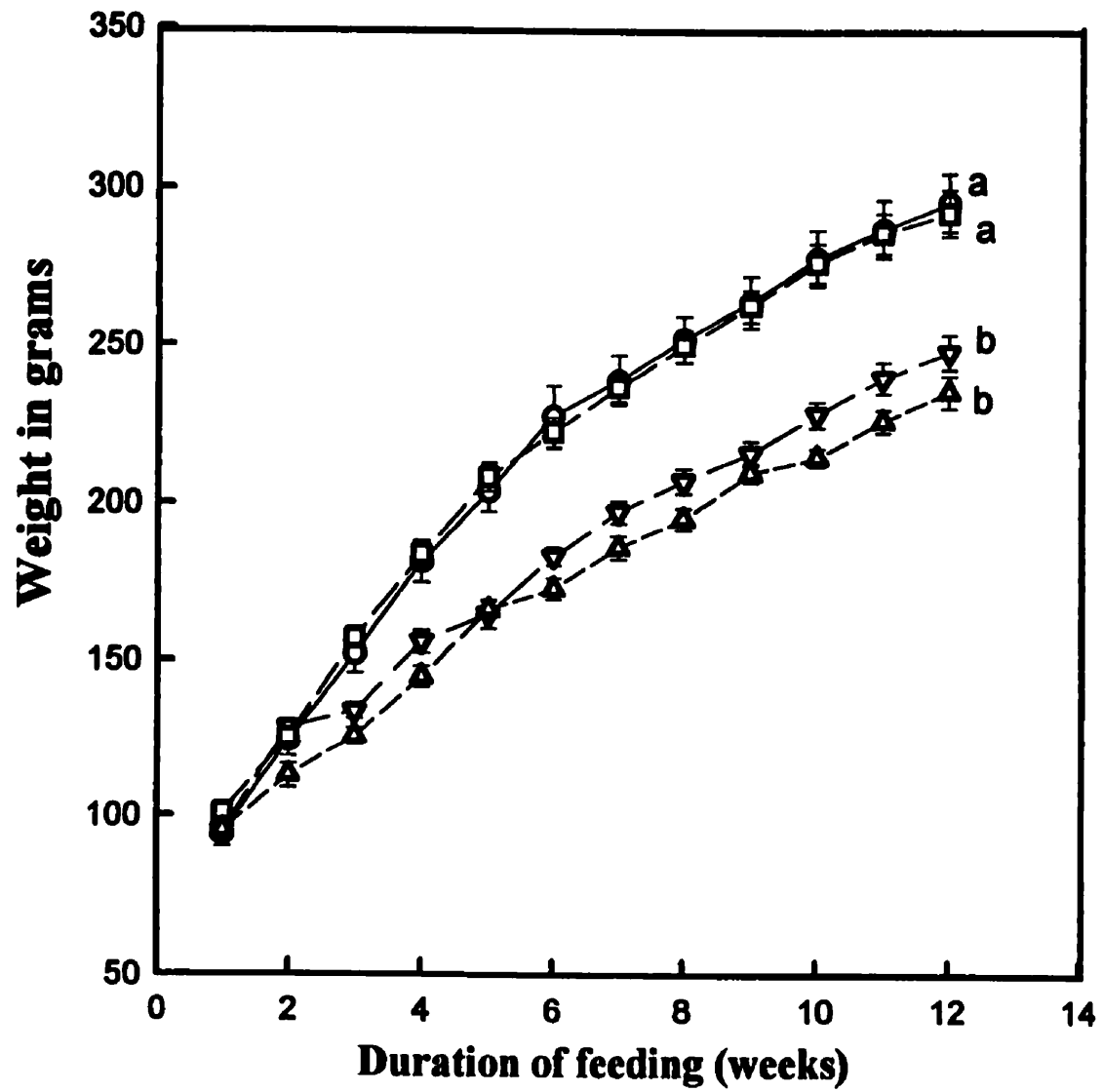
Figure 4.2 Average body weights (values are mean±SEM[bars]) of old F344 rats fed one of four diets, HF, HFER, LF, LFER for 12 weeks. Means not sharing a common superscript([a,b,c,d]) are significantly different (P ≤0.05, repeated measures ANOVA and Duncan's multiple range test).
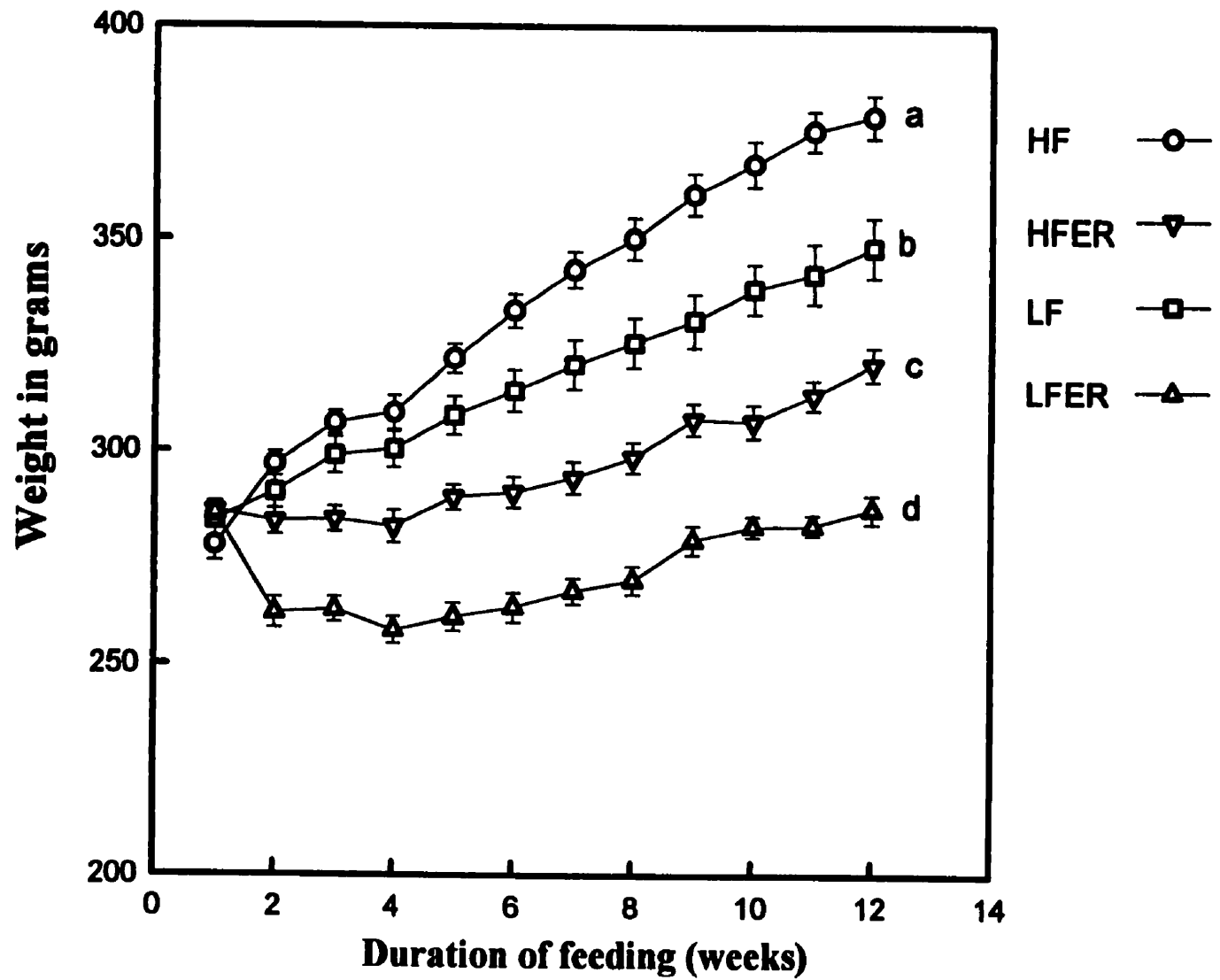
Table 4.2 Protein Kinase C (PKC) activity in young and old F344 rats fed one of four diets HF, HFER, LF, LFER for 12 weeks.

PKC ACTIVITY OF YOUNG AND OLD F344 RATS [1]			
DIET	Total PKC[1,2]	Cytosolic PKC[1]	Membranous[1] PKC
HF young	467.12±201.87	118.46±46.96	348.60±155.63
old	158.71±39.57	54.39±14.70	104.33±32.90
HFER young	374.94±130.71	73.47±28.94	301.55±105.31
old	148.27±38.37	50.78±6.53	97.48±33.79
LF young	373.69±169.96	105.80±36.70	268.10±142.59
old	145.73±26.01	45.49±9.01	80.24±27.99
LFER young	280.42±102.71	41.51±9.87	238.88±95.34
old	138.70±39.61	65.04±16.06	73.66±25.74

[1] Activity expressed as pmol Pi/min/mg protein, values are means±SEM.
[2] Total PKC represents cytosolic + membranous activity

Table 4.3 Protein Kinase C (PKC)two-way ANOVA P values

Two-way ANOVA *P* values			
Variable	Total[5] PKC	Membranous PKC	Cytosolic PKC
Fat[1]	0.5157	0.4703	0.5647
Kilojoules (KJ)[2]	0.5308	0.7338	0.1876
Age[3]	0.0081	0.0044	0.0856
FatxKJxAge[4]	0.9680	0.9951	0.4094

[1] Groups animals together by level of fat ([HF+HFER] and [LF+LFER]) regardless of restriction of kilojoules.
[2] Groups animals together as ad libitum (HF+LF) and restricted (HFER+LFER) regardless of level of fat.
[3] Groups animals together by age, regardless of diet.
[4] Determines the existence of an interaction of all variables (fat,KJ, and age)
[5] Total PKC represents cytosolic+membranous activity.

Colonic TK activity

TK activity was not consistently affected by dietary treatment in either of the cell

fractions (Table 4.4). However, TK activity does not appear to be as variable from one

animal to another as the PKC activity. It is worth noting that there was a trend for the old

animals to have lower total TK activity (membrane + cytosol activity) (two-way

ANOVA, $P \leq 0.0427$) (Table 4.5). (With the exception of the HFER and old HF groups,

all other diet groups expressed the highest TK activity in the membrane fraction. The

statistical analysis of percentage of cytosolic and membranous TK activity, relative to the

total activity, did not reveal any significant diet or age effects (data not shown). The

range of calculated TK activity values does appear to be compatible with previous

investigations in non-injected mucosa (Rao et al., 1993 and Singh et al., 1992)

PKC Isoform Expression

PKC α and γ were detectable in both the membrane and cytosolic fractions in all

groups (Figures 4.3). PKC α was detected in the positive control homogenates (HeLa

cells for PKC α, Jurkat for PKC γ, Transduction Laboratories, Lexington, Kentucky),

indicating that the antibody had sufficient titre and binding affinity. The isoform give a

single band at approximately 80 kDa. Particular isoform expression did not reveal any

significant difference between the diet or age groups (Figures 4.4, 4.5). This held true for

both isoforms. However, it is interesting to note that although not significant, there was a

trend for the younger animals to have a higher expression of PKC α and γ than the older

animals. This parallels the trend observed in the analysis of PKC activity. Conversely, the

Table 4.4 Tyrosine Kinase (TK) activity in young and old F344 rats fed one of four diets HF, HFER, LF, LFER for 12 weeks.

TK ACTIVITY OF YOUNG AND OLD F344 RATS [1]			
DIET	Total TK[1,2]	Cytosolic TK[1]	Membranous[1] TK
HF young	87.88±13.42	31.02±7.37	56.86±13.43
old	59.11±13.45	34.03±9.32	25.09±6.08
HFER young	62.04±2.87	29.68±8.39	25.28±7.40
old	46.10±12.46	16.55±9.08	25.44±5.53
LF young	59.06±23.06	23.42±9.36	31.87±12.09
old	51.44±6.45	18.66±2.41	30.65±7.37
LFER young	79.57±13.15	36.58±8.57	42.98±14.38
old	49.84±7.46	17.40±4.56	28.72±10.83

[1] Activity expressed as pmol Pi/min/mg protein, values are means±SEM.
[2] Total TK represents cytosolic + membranous activity

Table 4.5 Tyrosine Kinase (TK) two-way ANOVA *P* values.

Two-way ANOVA *P* values			
Variable	**Total TK[5]**	**Membranous TK**	**Cytosolic TK**
Fat [1]	0.6958	0.9598	0.4813
Kilojoules (KJ) [2]	0.6085	0.4744	0.7483
Age [3]	0.0427	0.1328	0.1204
FatxKJxAge [4]	0.5090	0.3238	0.3676

[1] Groups animals together by level of fat ([HF+HFER] and [LF+LFER]) regardless of restriction of kilojoules.
[2] Groups animals together as ad libitum (HF+LF) and restricted (HFER+LFER) regardless of level of fat.
[3] Groups animals together by age, regardless of diet.
[4] Determines the existence of an interaction of all variables (fat, KJ and age)
[5]Total TK represents cytosolic+membranous activity.

Figure 4.3 Membranous and cytosolic PKC α isoform expression in young and old rats. Protein bands were detected by enhanced chemiluminescence and developed on Kodak x-OMATAR film.

PKC α as Detected by Western Blotting Techniques

Cytosolic Fraction

80 kDa

Young   Old

Membranous Fraction

80 kDa

Young   Old
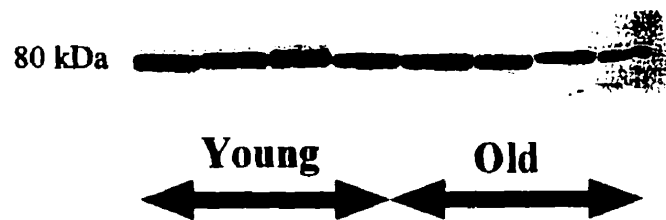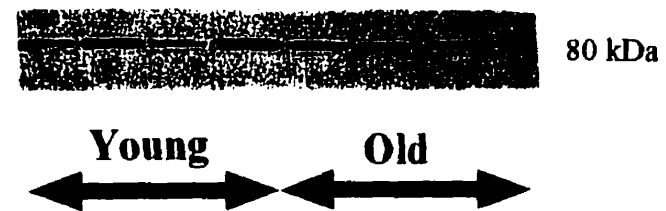
Figure 4.4 Average normalized area (values are means ±SEM[bars]) of protein bands for

PKC α expression in membranous and cytosolic cell fractions in young and old rats.
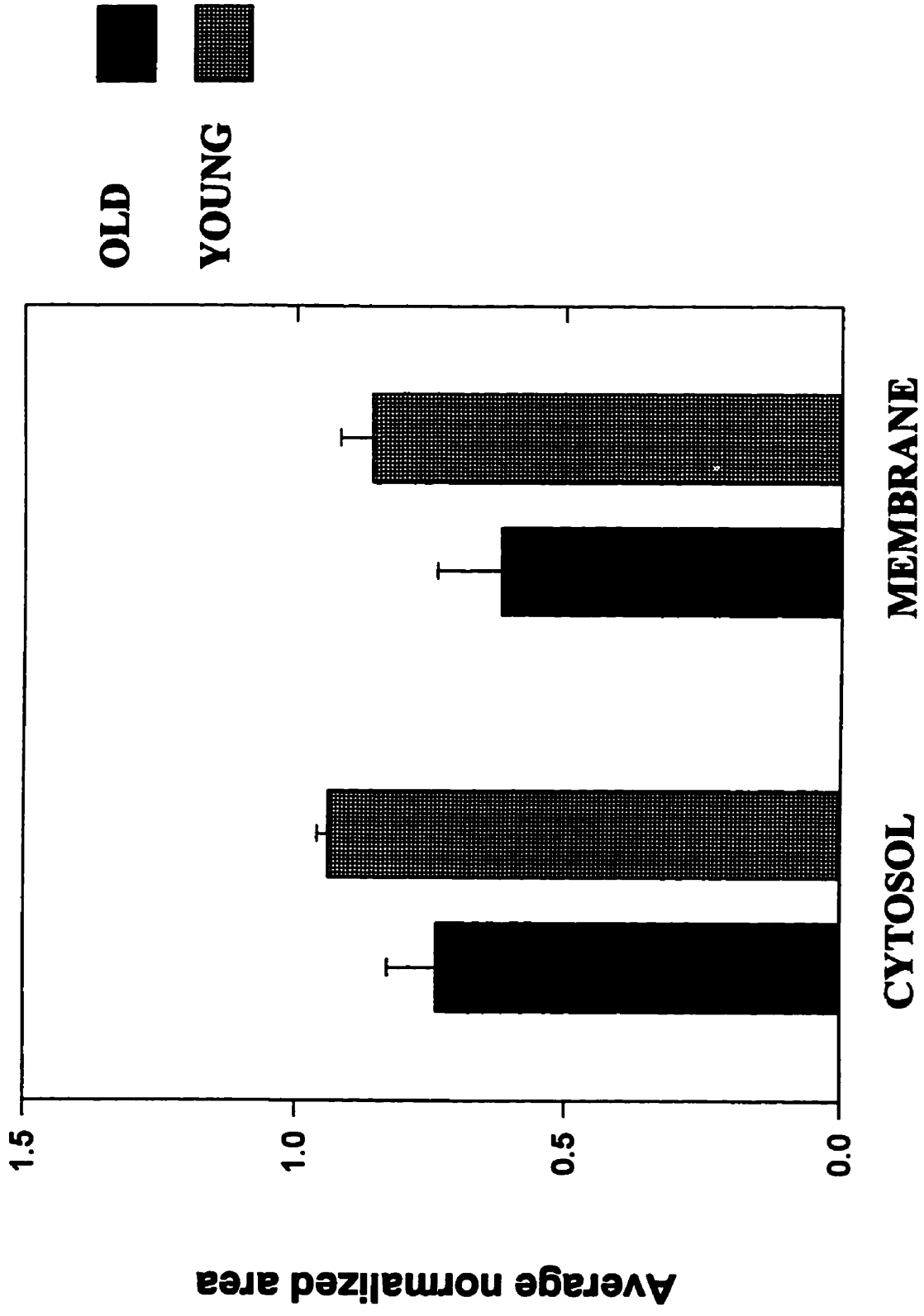
Figure 4.5 Average normalized area (values are means ±SEM[bars]) of protein bands for

PKC γ expression in membranous and cytosolic cell fractions in young and old rats.
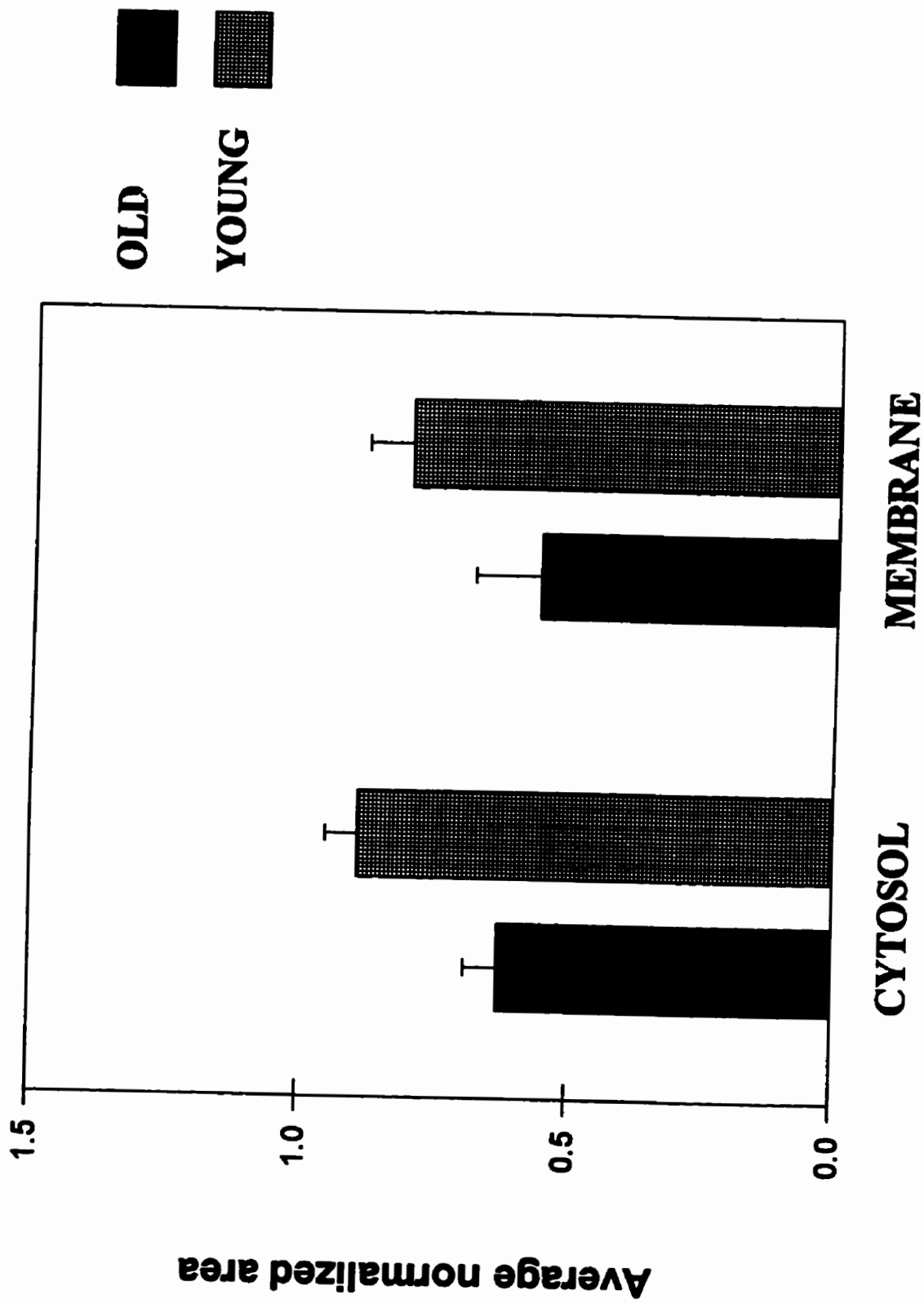
cytosolic fractions exhibited more intense signals for both isoforms as compared to the membrane fractions yet the enzyme activity was generally higher in the membrane fraction.

4.4 Discussion

The present study was the first to examine both PKC and TK in non injected colonic mucosa in an ER environment. The convergence of these pathways has been postulated in signal transduction event at the receptor membrane level (Ullrich and Schlessinger, 1990) and further downstream (via the RAS signal transduction pathway) involving cell functions at the nuclear level (Wiesmuller and Wittinghofer, 1994).

The main finding of this study demonstrate age related changes in rat colonic mucosa in both PKC and TK activity, with the younger rats exhibiting higher activity than the older animals. Although previous studies have demonstrated the ability of energy restriction to significantly inhibit age related changes in several cellular functions (Kalant et al., 1988; Duffy et al., 1989), ER within the older animal group (high or low fat) did not affect either of the enzymes. Dietary restriction was implemented only at the level of 20%, which may be classified as a mild level of restriction in the context of animal model studies which have restricted energy at levels as high as 60% of the ad libitum group (Breese et al., 1991). Therefore, this level of restriction may have prevented further changes in enzyme activity.

Dietary fat did not significantly affect the activity of either enzyme. However the membrane associate PKC activity exhibited a trend for the high fat animals to have higher activity than the low fat animals in both age groups. It is well documented that PKC is primarily located in the cytosolic fraction of the cell, until it is activated by various signals

(including lipid metabolites) it then translocates to the membrane where it becomes active (Nishizuka, 1992). However, it is now recognized that PKC may also translocate to the nucleus (Simboli-Campbell et al., 1994) where it may affect various proteins involved in transcription factors (Blobe et al., 1994). Therefore it would seem logical that the amount of available lipid in the diet, and perhaps total lipid content in cellular membranes may change the function of this membrane associated, lipid activated enzyme.

However, a recent study conducted in mice skin cells, ER significantly reduced PKC activity yet the total lipid and phospholipid composition was not altered (Kris et al., 1994) Interestingly ER significantly increased the level of diacyl-glycerol, a membrane lipid derived cofactor of PKC activation, in the skin cells (Kris et al., 1994). In rat colonic mucosa, HF diets have been demonstrated to exert little effect on lipid membrane composition with the exception of an increased phosphatidyl choline/phosphatidyl ethanolamine ratio in relation to low fat diets (LaFave et al., 1994). Such evidence suggests that HF and ER may modify PKC activity through a pathway other than alterations in lipid composition. Although we observed a trend for the high fat animals to have higher activity than the low fat groups, this was not statistically significant. This could have been the result of the large in between animal variance.

The expression of PKC α in all groups was expected because it has been identified as one of the major isoforms present in colonic mucosa (Davidson et al., 1995). Expression of PKC γ was unexpected considering it has been reported to exist only in the central nervous system (Hug and Sarre, 1993). However, the mucosal scraping prepared in this study may contain several different types of colonic cells other than the epithelial

cells.   The scrapings may have also included cell types such as stromal cells. Expression

of PKC isoforms in distinct populations of colonic cells has not been systematically

examined. Therefore, the expression of PKC γ in the present study may reflect the

existence of this isoform in a particular colonic cell type. In addition, the source of the

antibody and its potential cross-reactivities with other isoforms may also create

discrepancies in the isoforms detected from one study to another. Expression of PKC α

and γ isoforms reflect the amount of the isoform at the protein level where as the

measurement of PKC activity reflects the functional ability of those proteins as enzymes

capable of being activated by calcium and DAG.   Both methods detected variability within

the diet groups and cellular fractions, yet both identified similar trends for the younger

groups to exhibit higher levels of expression and activity as compared to the older groups.

It is interesting to note the more intense staining of the isoforms in the cytosolic fractions

as compared to the membrane fractions yet the enzyme activity exhibits the opposite trend.

These observations suggest that the majority of PKC remained in the cytosolic but that

the translocated membranous PKC was the active form.   Immunoblotting with a

polyclonal Pan-PKC antibody, which would reflect the amount of all isoforms at the

protein level, may provide a more accurate insight towards the relationship between

isoform expression and enzyme activity.

Carcinogen treatment of rat colonic mucosa significantly enhances the activity of

both TK (Arlow et al., 1989; Rao et al., 1993; Singh et al, 1992) and PKC (Craven and

DeRubertis, 1992b).   The majority of the diet intervention studies assaying TK fed a

particular diet for a short period of time and measured activity immediately and up to 1

week after injection of carcinogen (Arlow et al., 1989; Rao et al, 1993). The responses of the normal colonic mucosa to diets of varying compositions implemented over several weeks is not well established. Contradictory to the findings of the present investigation, colonic TK activity has been shown to increase with age (Majumdar and Tureaud, 1992). However, the ages of the old animals between the two studies are quite different which in part may account for the observed discrepancies.

The dietary treatments implemented in the present study may in fact have more of an impact in a carcinogen treated colon. Conversely, the changes in diet may have in fact affected the activity of these enzymes at the early stages of feeding, and may have adapted to these dietary changes over the duration of the experiment. In the present study, the response of colonic PKC and TK to exposure of different diets for 12 weeks was studied. This duration is considerably longer than those used in other studies. Therefore, this may in part explain the lack of significant differences among the groups. A non-carcinogen treated protocol in conjunction with changes in the level of dietary fat and energy provides a base-line for measuring such enzymes for future studies in which similar dietary protocols are to be implemented in conjunction with carcinogen treatment.

5. THE EFFECTS OF ENERGY RESTRICTION (20 AND 40%) IN HIGH
FAT DIETS ON DISTAL AND PROXIMAL COLONIC PROTEIN
KINASE C (PKC) ACTIVITY AND PROLIFERATING CELL NUCLEAR
ANTIGEN EXPRESSION IN MALE SPRAGUE DAWLEY RATS

5.1 Introduction

Structural and functional differences in the regions of the colon are a topic that

many scientific investigations do not address when exploring the realm of colon

carcinogenesis. The structure of the proximal and distal portions of the colon are very

different even when examined with the naked eye. Faecal matter entering the colon at the

proximal end is very different from the faecal matter leaving the distal end, suggesting that

the regions of the colon are exposed to very different environments and may even harbour

very different profiles in functions involved in nutrient uptake and transport.

Differences in proximal and distal cell proliferation markers have been identified

previously (Steinbach et al., 1993). However, the measurement of cell proliferation itself

has been subject to criticism based on its reproducibility (Roe et al., 1996). Differences in

methodology used to measure proliferative indices and the variation in assessment from

one lab to another may account for discrepancies in the trends observed in proximal versus

distal proliferative indices (Roe et al., 1996).

Colonic protein kinase C (PKC) activity has not been assessed in the varying

regions of the colonic mucosa. However, a recent study (Davidson et al., 1995) has

demonstrated differences in the expression of PKC isozymes in the proximal and distal

regions of the colon in rats fed varying types of fiber and fat. Consequently, PKC

membrane activity has been positively correlated with proliferative parameters in the rat

colonic mucosa, suggesting that PKC plays an important role in cell growth and

differentiation (Chapkin et al., 1993).

The previous study (Chapter 4) did not reveal any significant effects of 20% ER on

PKC activity however this level is considered mild and may not have been significant

enough to alter enzyme activity. Therefore, based on the previously discussed evidence,

the objectives of the present investigation were to determine the effects of 20 and 40% ER

in high fat corn oil diets on distal and proximal; 1) PKC activity; 2) cell proliferation; 3)

expression of PKC α; and 4) the relationship of these parameters to each other.

5.2 Material and Methods

Animal care and diets:

The animals were housed and cared for according to Chapter 4. The diets were

based on a semi-synthetic AIN-76 standard diet with modifications as specified in Table

5.1.

Table 5.1
Composition of HF(high fat), HF20 (high fat 20% energy restricted), HF40 (high fat 40% energy restricted) diets.

COMPONENT	%COMPOSITION		
	HF	HF20	HF40
	g/100g		
Casein	23.00	23.00	23.00
D-L Methionine	0.30	0.30	0.30
Corn Starch	33.75	31.46	29.18
Dextrose	8.52	8.52	8.52
Corn oil	23.00	23.00	23.00
Cellufil	5.90	7.08	8.26
AIN-76 Min.* Mix.	4.11	4.93	5.75
AIN-76 Vit.* Mix.	1.18	1.42	1.65
Choline Bitarate	0.24	0.29	0.34

*Refer to appendix D for content.

Study Design:

Thirty weanling male Sprague-Dawley rats (University of Manitoba Central Breeding) were used. After one week of acclimatization on standard lab chow, the animals were housed in single cages and randomly allocated 10 per group into 3 experimental groups. These groups were fed one of the HF, HF20 or HF40 dits. Diets within each fat classification were isocaloric. Therefore, an ER at 20 and 40% was accomplished by feeding the ER groups 80 and 60%, respectively, of the average daily intake of the HF counterparts. Initial and weekly body weights were recorded. After four weeks of feeding all animals were killed, the colons were removed, flushed with phosphate buffered saline (PBS), slit from the caecum to the anus and placed on a cooled surface at 4°C. Two 1 cm² sections was taken from the distal and proximal regions of each colon and fixed flat in between filter papers in 70% ethanol. Each colon was then divided into two sections, distal (0-8 cm) and proximal regions (8-12 cm) and scraped on a cold plate held at 4°C. All sections were placed in sterile cryovials and frozen immediately in liquid nitrogen and stored at -80°C.

Proliferating Cell nuclear Antigen expression

PCNA expression was determined according to the method described in Chapter 3 and assessed in 5 colons from each diet group.

Extraction, Separation of Colonic Cytosol and Membrane Fractions for PKC activity and Western Blotting

The fractions designated for PKC activity and western blotting procedures were

prepared according to the protocol in Chapter 3.

PKC Assay

The PKC assay was conducted on the membrane and cytosol fractions utilizing the Amersham kit as described in Chapter 3.

Protein Analysis

Protein content was determined by the method described in Chapter 3.

Detection of Antigens by Western Blotting

The expression of PKC α was determined in the proximal and distal cytosol and membranous fractions according to the method described in Chapter 3.

Statistical Analysis

Statistical analysis of PCNA, PKC activity, and PKC isoform data was carried out using analysis of variance (ANOVA) in conjunction with Duncan's Multiple Range Test. Body weights were analysed by repeated measures ANOVA. Correlations were determined using Pearson's and Spearman's correlation coefficients. All tests were conducted using the SAS statistical software package for microcomputers (SAS Institute Inc., Cary, NC). A P value ≤ 0.05 was considered significant.

5.3   **Results**

Body weight

Body weights of the three groups at the start of the experiment were not significantly different (Figure 5.1). By the first week of feeding their respective diets, the HF group weighed more than the HF20 group which weighed more than the HF40 group. These differences remained significant for the duration of the experiment.

Figure 5.1 Average body weights of male Sprague-Dawley rats fed one of three diets,

HF, HF20, HF40, over the duration of 4 weeks. Values are means ±SEM.

WEIGHT IN GRAMS (y-axis): 100, 150, 200, 250, 300, 350, 400, 450, 500

WEEKS ON EXPERIMENTAL DIET (x-axis): 0, 1, 2, 3, 4

Legend:
- ● HF
- ■ HF20
- ▲ HF40
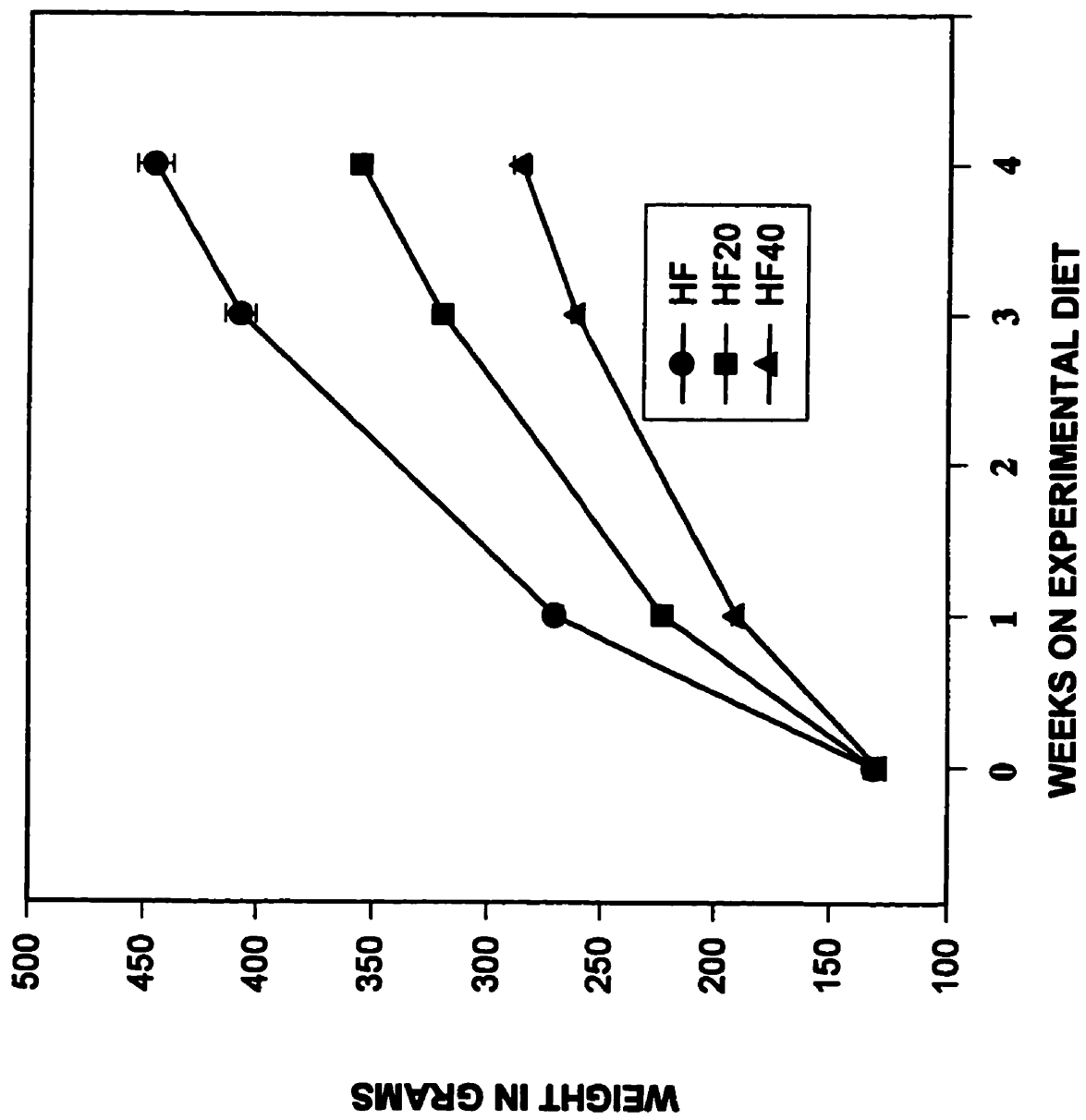
Colonic PKC activity

PKC activity was assessed after 4 weeks of feeding and is detailed in Table 5.2.

Cytosolic and membranous activity within all diet groups was quite variable.

Therefore, statistical analysis did not reveal any significant differences. However,

there was a trend for the activity to increase with increasing level of energy restriction,

with the specific order of HF < HF20 < HF40. This trend was observed in both

regions and in both cellular fractions. The cytosolic activity was lower than the

membranous activity. The region of the colon (proximal versus rectal) did not exhibit

any significant differences in activity.

PKC α Expression

As shown in Figure 5.2, PKC α was present in the rat colonic mucosa

membrane fractions as detected by immunoblotting. PKC α was detected in the

positive control homogenate (HeLa cells, Transduction Laboratories, Lexington

Kentucky), indicating that the antibody had sufficient titre and binding affinity. The

isoform gives a signal band at approximately 80 kDa. Within the membrane fraction,

the HF20 group exhibited a significantly higher expression of PKC α in the distal

region than in the proximal region (Figure 5.3). Although not significant, there was a

trend for the distal regions to exhibit higher expression than the proximal regions.

PCNA Expression

The proliferative zone of the colonic crypt probed with anti-PCNA was not

affected by the region of the colon within a diet group (Table 5.3). Among the diet

Table 5.2 Protein Kinase C (PKC) activity in male Sprague-Dawley rats fed one of three diets HF, HF20, HF40 for 4 weeks.

DIET	Total Proximal	Proximal cytosol	Proximal membrane	Total Distal	Distal cytosol	Distal membrane
HF	203.23 ±83.54	0.36 ±0.36	202.87 ±83.71	161.17 ±76.10	8.20 ±5.84	151.77 ±71.31
HF20	369.84 ±99.86	73.77 ±69.31	296.06 ±100.26	130.81 ±17.12	11.97 ±6.73	118.78 ±21.71
HF40	716.82 ±357.74	84.96 ±72.39	631.86 ±342.24	728.25 ±498.78	104.62 ±70.62	623.64 ±498.02

[1] Activity expressed as pmol Pi/min/mg protein, values are means±SEM.

[2] Total PKC represents cytosolic + membranous activity

Figure 5.2 Proximal and distal membranous PKC α isoform expression in rats fed the

HF20 diet for 4 weeks. Protein bands were detected by enhanced chemiluminescence

and developed on Kodak x-OMATAR. PKC α positive control was HeLa cell line

(Transduction Laboratories, Lexington, Kentucky).

PKC α as Detected by Western Blotting Techniques

HF20 Membranous Fraction

Positive Control

80 kDa
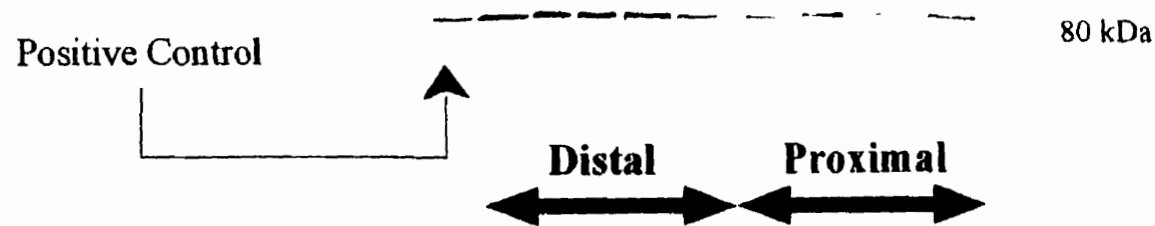
Distal   Proximal

Figure 5.3 Average normalized areas of protein PKC α protein bands in membranous

proximal and distal colonic regions in rats fed one of three diets HF, HF20 and HF40

for 4 weeks. Values are means $\pm$SEM(bars). Means not sharing a common

superscript are significantly different (P $\leq$0.05, ANOVA and Duncan's multiple range

test).
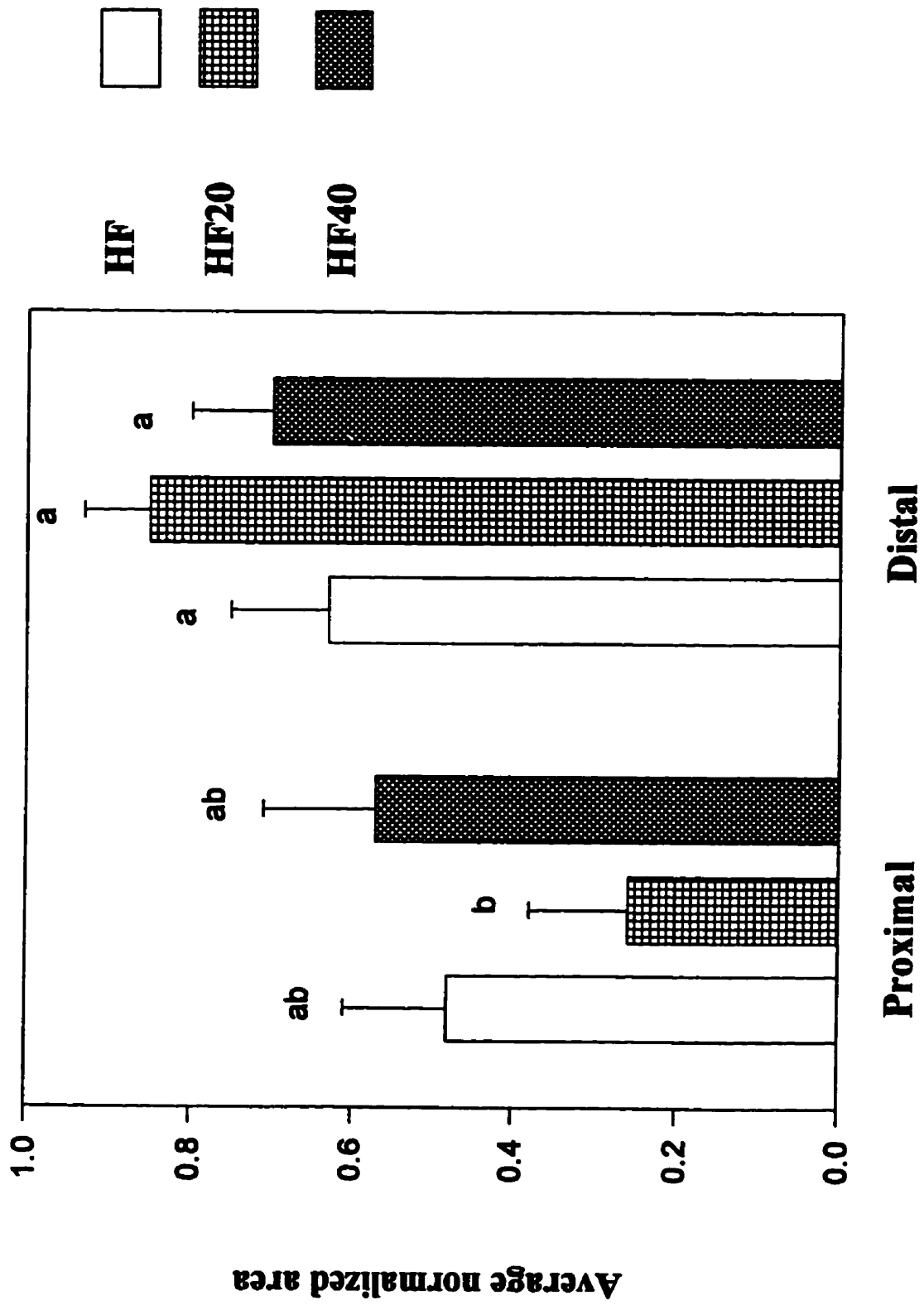
Table 5.3 Proliferating Cell Nuclear Antigen Expression (PCNA) in colonic proximal and distal sections in male Sprague-Dawley rats fed one of three diets, HF, HF20, HF40 for 4 weeks [1].

DIET n = 5/group	Proliferative Zone[2]		Labelling Indices[3]	
	Proximal	Distal	Proximal	Distal
HF	14.46±1.16 ^{ab}	11.94±0.22 ^{b}	33.62±1.28 ^{b}	36.08±1.26 ^{ab}
HF20	16.44±1.36 ^{a}	14.14±1.39 ^{ab}	43.88±3.52 ^{a}	38.42±2.08 ^{ab}
HF40	13.54±0.64 ^{ab}	13.40±0.54 ^{ab}	31.70±0.90 ^{b}	32.32±4.81 ^{b}

[1] Values are mean±SEM. Means in a column not sharing a common superscripts are significantly different ($p \leq 0.05$, ANOVA and Duncan's multiple range tests).
[2] Represents the highest labelled cell in the crypt.
[3] Labelled cells/100 cells in each group.

groups, the HF20 proximal region displayed a significantly higher proliferative zone than in the distal region of the HF group. Labelling indices were not affected by the region of the colon within the diet groups. Within the proximal region of the colon, the HF20 exhibited a higher labelling index than the HF and HF40 groups.

Correlation analysis did not reveal any significant relationships between PKC activity, PKC isoform expression or PCNA expression (data not shown).

5.3   Discussion

It has been previously demonstrated that PKC activity is significantly affected by the amount of fat in the diet (Chapkin et al., 1993). However, the effect of different levels of ER on colonic PKC activity in different regions of the colon is not well established.

The present investigation explored the effects of 20 and 40% ER in high fat diets on proliferative indices, PKC activity and PKC α isoform expression.   PKC activity was higher in the membranous fraction in all groups relative to the cytosol which has been previously demonstrated in previous studies (Craven and DeRubertis, 1992).   ER did not significantly modify PKC activity at either level nor were there any significant differences between the proximal and distal regions of the colon.   However, there was a trend for the ER rats to have higher PKC activity in both cell fractions and regions of the colon and possess higher PKC α in the membrane fraction of the distal colon.   Feeding an HF20 diet significantly increased the LI for PCNA in the proximal region of the colon. Such results may seem conflicting, considering the extensive evidence supporting ER as a tumor inhibitor and the role of elevated PKC and cell

proliferation in carcinogenesis. However a previous investigation by Craven and DeRubertis (1992b) demonstrated similar findings with higher cell proliferation, PKC activity, and PKC α mass in fasted compared to ad libitum fed rats.

It has also been substantiated that reduced PKC activity is observed in cells after sustained activation *in vitro* (Murray et al., 1987). Such evidence suggests that an observed reduction in PKC activity and PKC α expression may in fact be an indication of prior activation. Additional studies have suggested that the down-regulation of enzyme activity, induced by repeated application of phorbel esters, rather than an initial activation of PKC may be the main factor mediating tumor promotion (Hansen et al. 1990;Fischer et al., 1989). Therefore, the trend for the activity to increase with increasing level of ER may in fact be an indication of a lesser amount of prior activation in relation to the HF group. The steady state levels of PKC activity in the HF group may have been higher in relation to the ER groups and could be more prone to down regulation upon stimulation by the phorbol ester (PMA) present in the assay reaction mixture.

Another explanation may stem from the evidence suggesting that the PKC isoforms may in fact possess divergent roles in signal transduction (Hugg and Sarre, 1993). The levels of the varying isoforms may in fact be very different in the ER compared to the HF groups. The measurement of one isoform may not provide sufficient evidence to suggest that all isoforms are affected by ER.

In addition, PKC's are heterogeneous protein kinases that share a common requirement for phospholipids for activity, but possess different dependency on other

activators (Kiley and Jakens, 1994). The assay in the present study includes the known activators of PKC, Ca^{2+}, phosphatidyl-L-serine (PS) and a phorbel ester, PMA. The only cofactor identified for activation of the atypical PKCs (ζ, λ, ι) is PS. These atypical forms may in fact require additional activation by factors within the cell not presently identified. PKC ζ has also been identified in the rat colonic mucosa (Jiang et al., 1995).

Conflicting results have been reported regarding the relationship between colonic PKC activity and cell proliferation (Chapkin et al., 1993, Craven and DeRubertis, 1987;Craven and DeRubertis, 1992a; LaFave et al., 1994). However, these studies vary extensively in the type of dietary protocol employs, method of PKC activation and method of cell proliferation measurement. In the present study, the method used to measure cell proliferative indices was the expression of PCNA, which is considered to be a non-invasive method considering it circumvents the use of radioactive ($[^3H]$ thymidine) or cytotoxic (colchicine, bromodeoxyuridine) chemicals. This method is ideal when enzymatic and proliferative measurements are to be evaluated in the same colonic mucosa. The HF20 exhibited a higher proximal LI than the other groups. However, the proximal PKC activity, although not significant, was the highest in the HF40 group. PKC activity tended to increase with increasing level of restriction, whereas the LI tended to increase in the HF20 and remained similar in the HF40 in relation to the HF group. As in the study conducted by Craven and DeRubertis (1992a), the results do not support a relationship between increased PKC activity and increased cell proliferation.

It is interesting to note that the pattern of proliferative zones in the distal regions follows the pattern of PKC α isoform expression in the distal region. For both measurements the order from highest to lowest was HF20 > HF40 > HF. Conversely the LI was the highest in the HF20 in the proximal region, yet this diet exhibited the lowest level of PKC α. Such observations suggest that the level of this isozyme may be indicative of the range of proliferation and not the LI within the colonic crypt.

In summary, these results suggest that ER may affect colonic PKC activity in non-carcinogen treated colonic mucosa without any regional disparities. Whether PKC activity relates to the proliferative state of a particular tissue is not well established and requires further clarification. Findings of the present study do not support the contention that increased PKC activity represents an increased number of proliferating (cycling) cells. PKC activity, and its relation to cell proliferation and expression of several PKC isozymes in the malignant transformation of colonic cells may be further elucidated in carcinogen treated colons. In addition, the role of increased PKC activity may need further exploration in order to determine whether or not it is positively associated with increased malignant potential.

6. MODULATION OF THE EARLY STAGES OF COLON CARCINOGENESIS BY ENERGY RESTRICTION AND DIETARY FAT IN MALE F344 RATS

6.1 Introduction

ER has been associated with decreased incidence of spontaneous and chemically induced tumors in various animal organs including chemically induced colon tumors (Klurfeld et al., 1987; Kumar et al., 1990; Reddy et al., 1987). These studies have demonstrated that ER is effective in inhibiting the development of colon tumors when introduced in the early stages of carcinogenesis and continued until the time of tumor development. Consequently, whether ER will be effective in retarding the growth of early or preneoplastic lesions remains elusive.

Aberrant crypt foci (ACF) are identified in carcinogen-treated rodent colons and human colonic mucosa (Pretlow et al., 1991; Roncucci et al., 1991), and purported to be putative preneoplastic lesions (Bird, 1987). Several studies substantiate this hypothesis (McLellan and Bird, 1988a,1988b; McLellan et al., 1991). ACF with varying growth features are reported to occur in a rat colon several weeks after a single injection of a colon carcinogen (Bird, 1987; McLellan and Bird, 1988a) presumably representing preneoplastic lesions at different developmental stages.

The ongoing research in our laboratory focuses on the evaluation of the ACF system to identify modulators of colon carcinogenesis as well as the use of this system to study growth regulation of the stepwise development of colon cancer by diet. In the present study we investigated the ability of the ACF system to predict the tumor

enhancing or inhibitory effect of a high corn oil or ER diet , respectively, as early as 4 weeks after carcinogen administration. Therefore, the two specific objectives of the present study were; 1) to assess if dietary fat and ER affect early stages of colon carcinogenesis in an independent manner; 2) to assess the value of the ACF system and proliferative indices in the identification of these dietary variables as tumor promoters or inhibitors and; 3) determine if diet elicits different responses in the distal vs. the proximal colon.

6.2   Materials and Methods

Animals

Forty eight male weanling F344 rats (Charles Rivers) were used. Animals were housed in suspended stainless steel wire bottom cages with sawdust bedding pans approximately 10 cm below the cages. Housing facilities were programmed for a 12 hour light-dark cycle. All animals were allowed free access to standard rodent laboratory chow and water as an acclimatization measure for two weeks (12 days). Care of the animals was in accord with the guidelines of the Canadian Council on Animal Care.

Carcinogen

Azoxymethane (AOM) (Sigma) a carcinogen known to induce colonic tumors, was dissolved in a fresh 0.9% saline solution one hour prior to injection. After the acclimation period all animals were injected s.c. with AOM for two weeks (15mg/kg body weight once weekly).

Diet

Semi-purified diets were based on the AIN-76A diets described in chapter 4. Animals were randomly allocated to one of four diets (n= 12/group) immediately after final injection and placed in single cages. These groups received four dietary regimes, the AIN-76A high fat (HF), high fat energy restricted (HFER), low fat (LF) and the low fat energy restricted (LFER) diets. Feeding protocols were followed as described in Chapter 4. Body weights were determined weekly thereafter until the termination dates at 4 (n= 24/group) and 8 (n= 24/group) weeks.

Quantification of Aberrant Crypt Foci.

All animals were killed, colons removed and ACF characteristics determined according to the method described in Chapter 3. Specifically, the ACF characteristics analysed included the total number, average crypt multiplicity and the number of small (1-2 crypts/focus at week 4, 1-3 crypts/focus at week 8), medium (3-4 crypt/focus at week 4, 4-6 crypts/focus at week 8) and large ACF (>4 crypts/focus at week 4, >6 crypts/focus at week 8). For each rat the average crypt multiplicity was correlated with fat and energy intake for all groups.

Assessment of Proliferating Cell Nuclear Antigen Expression

Proliferating cell nuclear antigen expression (PCNA) was determined according to the method described in Chapter 3 and assessed in 6 animals per group.

Statistical Analysis

SAS statistical software for microcomputers was used for all statistical analyses. Statistical analyses of ACF data and proliferative indices were performed by analysis of variance (ANOVA) and Duncan's multiple range test ($P \leq 0.05$). This data was further

analysed using a two way ANOVA in order to determine the main effects of fat, ER and possible fatxER interactions (fatxER). Body weights and diet intake were analysed by repeated measures of analysis of variance. Correlation between two parameters was established using the Spearman correlation coefficient.

6.3 Results

At week 2 of feeding body weights (Figure 6.1) were significantly lower in the ER groups with HF weighing the most, followed by LF and HFER (no difference between them) and LFER with the lowest weight. This trend persisted until the final termination date at week 8. The average KJ/day and the average grams of fat consumed/day at week 4 and week 8 time points were significantly different between all groups (Table 6.1). Both the HFER and LFER groups consumed significantly less KJ/day and fat/day than their respective counterparts.

ACF were present throughout the length of the fixed colon with the majority in the distal region at week 4 and in the proximal region at week 8 (Figure 6.2). In all groups the number of ACF was higher in the proximal colon at week 8 than in week 4. However, the mean total of ACF per colon were not significantly different between any groups at week 4 or week 8 (data not shown).

At week 4 average crypt multiplicity of ACF was significantly lower in the LFER group compared to the HF group whereas the HFER and LF group crypt multiplicities were not different (Figure 6.3). Fat was identified as the main variable affecting week 4 average crypt multiplicity (two-way ANOVA P=0.038) among the groups. Further analysis of ACF, grouped according to their crypt multiplicity, revealed no effects of ER

Table 6.1 Mean daily intake of diet, energy and fat for male F344 rats fed one of four diets, HF, HFER, LF and LFER 4 and 8 weeks after injection.

		HF	HFER	LF	LFER
Diet g/day[1]	wk4	11.5±0.3[b]	9.5±0.0[c]	13.6±0.3[a]	11.0±0.0[b]
	wk8	11.4±0.4[b]	9.1±0.0[d]	12.7±0.4[a]	10.0±0.0[c]
KJ/day[2]	wk4	241.08±6.7[a]	199.65±0.0[c]	228.11±5.4[b]	184.59±0.0[d]
	wk8	238.57±7.1[a]	190.4±0.0[c]	212.62±6.7[b]	167.42±0.0[d]
Fat g/day[3]	wk4	2.7±0.1[a]	2.2±0.0[b]	0.7±0.0[c]	0.6±0.0[d]
	wk8	2.6±0.1a	2.1±0.0[b]	0.6±0.0[c]	0.5±0.0[d]

[1,2,3] Values are mean ± SEM (n= 10 rats/group)

Means in a row not sharing a common superscript are significantly different ($P \leq 0.05$, ANOVA and Duncan's multiple range test).

KJ = kilojoules.

Fig. 6.1 Average body weights of male F344 rats fed one of four diets (HF; high fat ,

HFER; high fat energy restricted, LF; low fat, LFER; low fat energy restricted). Values

are means ± SEM (n=12/group until week 4, n=6/group until week 8). Means not sharing

a common superscript at week 8 are significantly different (P≤0.05, Repeated Measures

ANOVA).
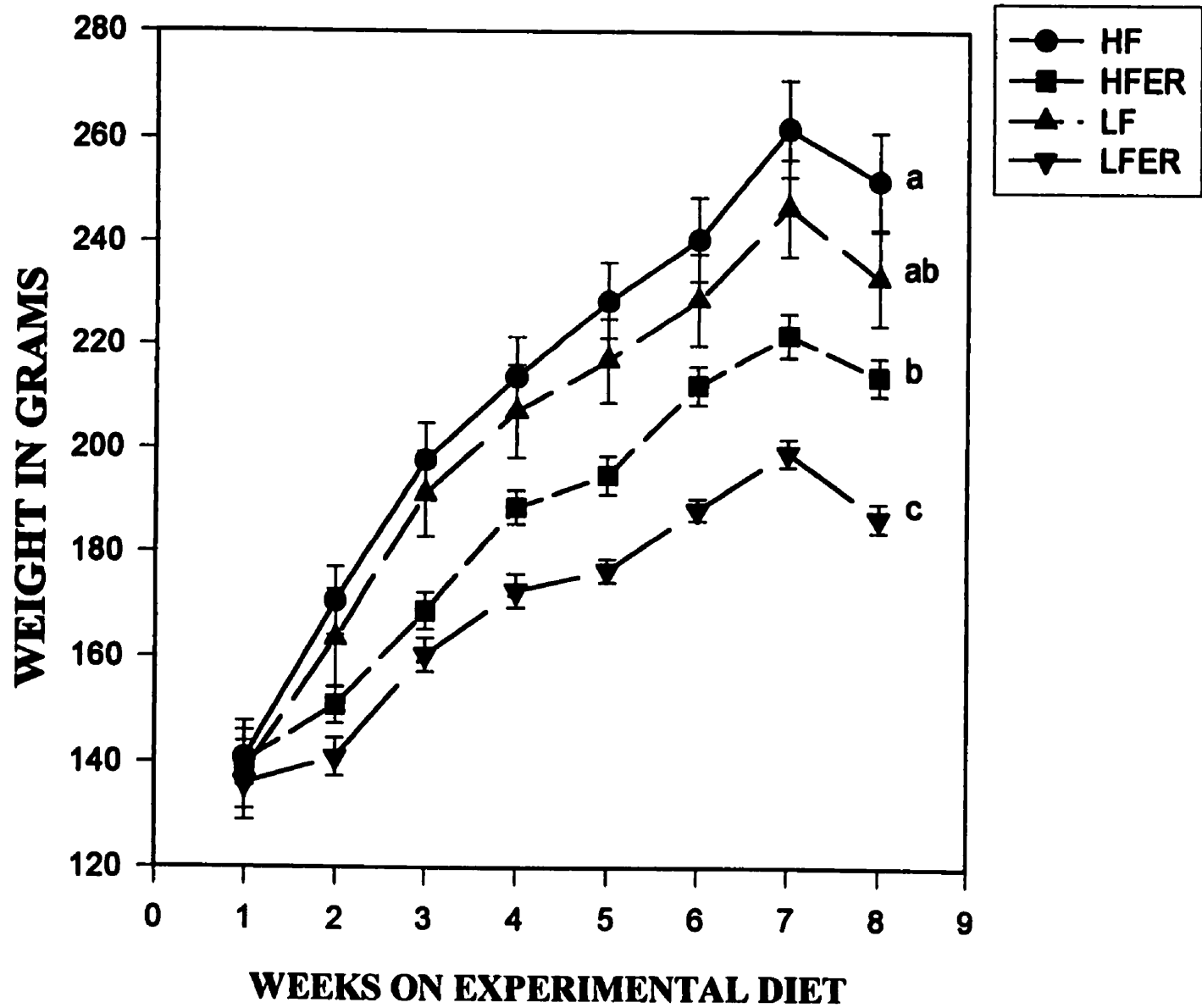
Fig. 6.2 Number of ACF (aberrant crypt foci) in the distal and proximal regions in the

whole colon/group at week 4 and week 8 in rats fed one of four diets (HF; high fat,

HFER; high fat energy restricted, LF; low fat, LFER; low fat energy restricted)

immediately after azoxymethane injection. Values are means ± SEM (n=6/group). The

asterisk denotes significant difference (P≤0.05, ANOVA) within the same region of the

colon within the same diet classification.
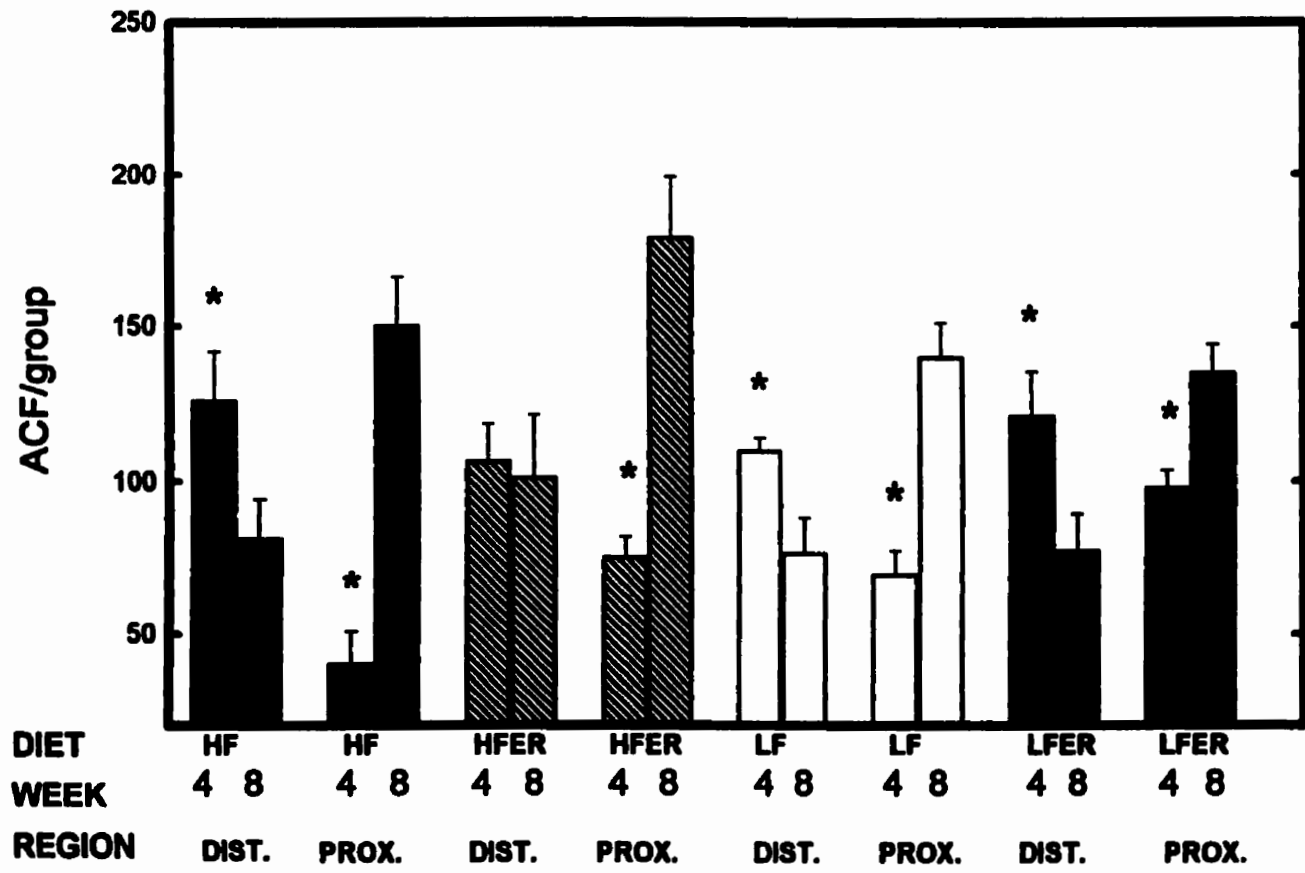
Fig. 6.3 Number of crypts/focus at week 4 and week 8 in rats fed one of four diets (HF; high fat, HFER; high fat energy restricted, LF; low fat, LFER; low fat energy restricted) immediately after azoxymethane injection. Values are means ±SEM, (n=6/group). Means not sharing a common superscript are different (P≤0.05, ANOVA and Duncan's multiple range test).
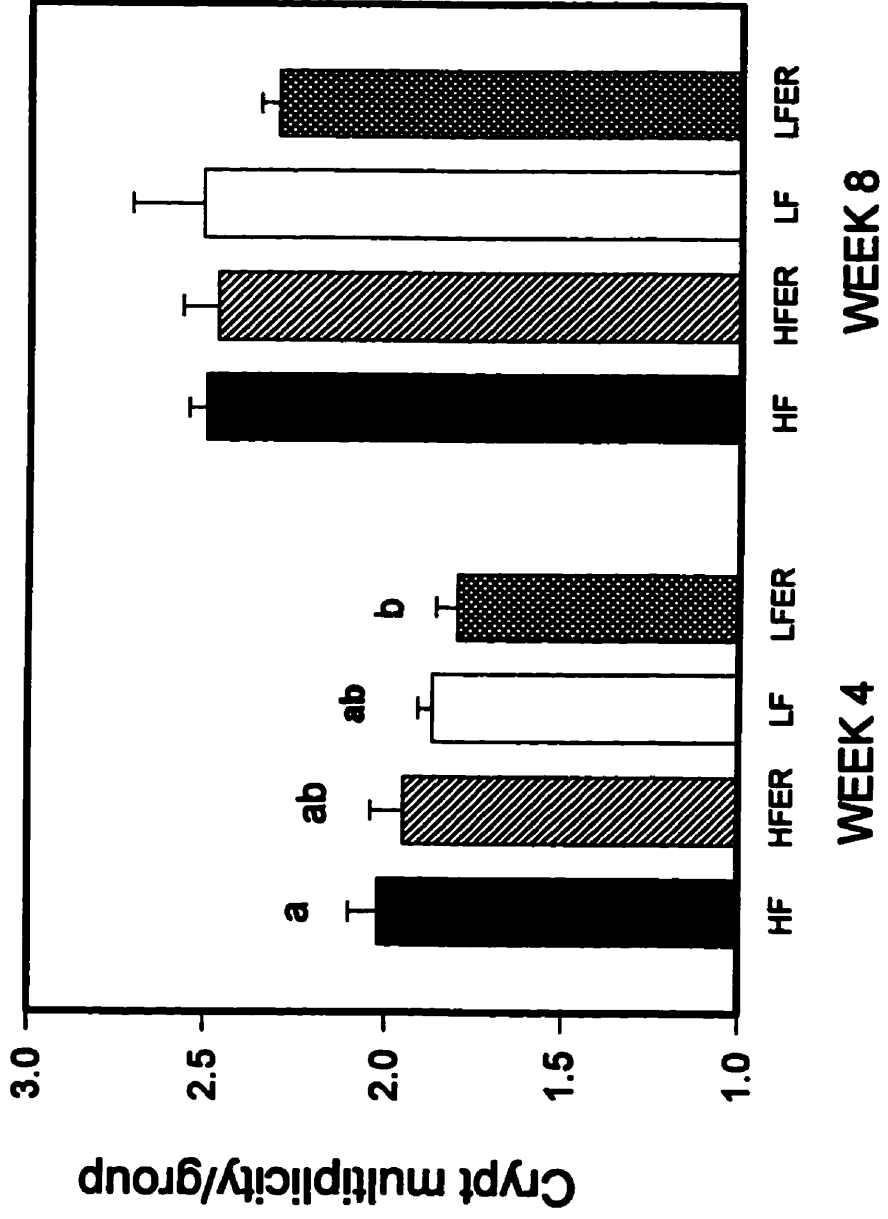
Fig 6.4. Number of small (1-3 crypts/focus), medium (4-6 crypts/focus) and large (>6 crypts/focus) ACF at week 8 in rats fed one of four diets (HF; high fat, HFER; high fat energy restricted, LF; low fat, LFER; low fat energy restricted) immediately after azoxymethane injection. Values are means ● SEM, n=6. Means in each classification not sharing a common superscript are significantly different ($P \le 0.05$, ANOVA and Duncan's multiple range test).
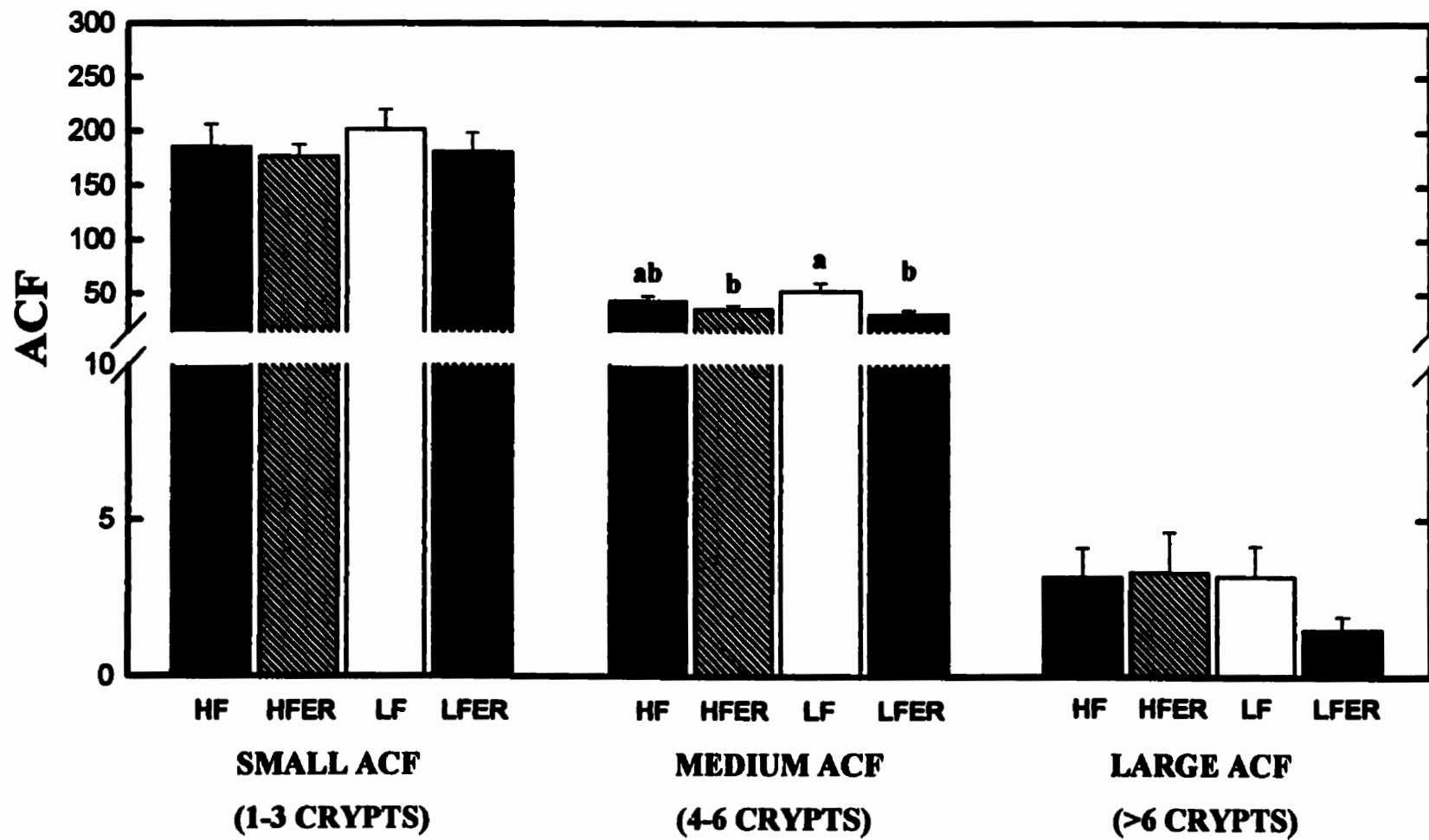
or fat on the number of small, medium and large ACF at week 4. However, by week 8 (Figure 6.4) the LFER and HFER groups exhibited fewer medium ACF in relation to the LF group, with HF not differing from any group. Two-way ANOVA identified ER as the main variable affecting the medium ACF category (P=0.0083).

Correlations between dietary variables and ACF characteristics were carried out on a per rat basis. The individual average intakes of energy (KJ) and fat (grams) per rat were correlated with their individual ACF characteristics. A positive correlation was noted between mean daily intake of fat and average crypt multiplicity of ACF at week 4 (r=0.49, P=0.013), and average crypt multiplicity values and mean daily intake of energy (KJ) (r=.46, P=.0263) and fat (grams) (r=.49, P=.017) at week 8.

PCNA labelling index (LI) at week 8 (Table 6.2) in the proximal section of the colon was not affected by dietary treatments. The distal LI were significantly lower in the LF and LFER groups relative to the HF group. LI values in the LFER were lower than their respective LF counterparts, however the HF and HFER values did not differ. ER (P=0.0087) and fat (P=0.0001) were both identified as variables significantly affecting the distal LI values. With the exception of the LFER group, the PCNA LI in the distal regions were significantly higher than those in the proximal region across all. Correlational analysis between ACF characteristics and proliferative indices in different regions of the colons revealed no strong positive associations.

Table 6.2

PCNA Labelling Index (LI)[1] in proximal and distal sections of colonic mucosa in male F344 rats fed one of four diets, HF, HFER, LF, LFER for 8 weeks.

Diet (n=6/ group)	Proximal Week 8	Distal Week 8
HF	35.9 ± 1.5^{a}	46.2 ± 1.6^{a} *
HFER	34.8 ± 3.6^{a}	$44.6 \pm 1.2^{a,b}$ *
LF	32.7 ± 3.6^{a}	41.1 ± 1.2^{b} *
LFER	34.2 ± 2.7^{a}	34.2 ± 1.5^{c}
Two-way ANOVA		
FAT^{2}	0.5391	0.0001
ER^{2}	0.9647	0.0087
$FATxER^{2}$	0.6678	0.0778

[1] Means ±SEM of PCNA (proliferating cell nuclear antigen) labelled cells/100 cells in each group.
Means in a column not sharing a common superscript are significantly different ($P \leq 0.05$, ANOVA, and Duncan's multiple range test).
*Depicts distal means which are significantly different from their proximal counterparts within each dietary group.
[2] P values.

6.4   Discussion

In the present investigation the effect of ER and a high corn oil diet on the early stages of colon carcinogenesis was studied by employing an experimental protocol used in previous tumor incidence studies (Kumar et al., 1990).   Aberrant crypt foci and colonic proliferative indices were used as the end points.   The main findings were that the effect of a high fat diet affected the crypt multiplicity of ACF within 4 weeks of feeding.   The effect of ER was significant at week 8 and was negatively correlated with the average crypt multiplicity of ACF.   At week 4, increased crypt multiplicity was significantly correlated with increased fat intake and negatively correlated with decreased energy intake.   ER was effective in reducing the number of cells exhibiting proliferative cell nuclear antigen in the distal region but not in the proximal region.   This effect was most pronounced among the animals fed a low fat diet.

The ACF system provides the unique opportunity to quantify preneoplastic changes occurring in colonic mucosa by a simple method.   Growth features of ACF which can be measured topographically are, their distribution, total number, size of each ACF (area occupied by each focus) and the number of crypts present in each ACF (crypt multiplicity).   The quantification of the number and growth features of ACF allows for comparison of the preneoplastic state among different groups.   Within two weeks after a single injection of a carcinogen, the majority of ACF consist of a single crypt (McLellan et al., 1991).   As time progresses, ACF exhibit varying crypt multiplicities and are known to harbour genotypic and phenotypic atypia (McLellan et al., 1991 and Stopera et al, 1992,).   Previous studies have suggested that average crypt multiplicity of ACF, or the number of

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ACF with higher crypt multiplicity in a group, is more sensitive in predicting the tumor

modulating ability of a compound than the number of ACF. Determination of the growth

features of ACF at more than one time point may indicate whether the disease process is

accelerated or impeded and whether the growth modulator under investigation is affecting

a population of ACF with specific growth features. Consequently , enumeration of the

number and growth features of ACF has been used successfully to identify

chemopreventive agents (Pereira, and Khoury, 1991 and Wargovich et al., 1996).

In the present study, the total number of ACF was not valuable as the end point in

predicting tumor modulating ability of a high fat or energy restricted diet. Crypt

multiplicity was the most sensitive measure, of ACF growth characteristics, in determining

the effects of fat and ER. The food consumption data for each individual animal allowed

us to evaluate whether there was a correlation between intakes of fat or energy and

growth features of ACF. The ability of a high or low fat diet with or without energy

restriction to modulate growth features of ACF was analysed by groups as well as by rat

to see correlations between fat or energy intake and ACF growth features. Such

correlations provided substantial evidence supporting previous findings that individual fat

and energy intake may significantly alter the disease process. In addition these findings

provide support to the contention that the effect of fat is separable from the ER effect.

The analyses of dietary variables and growth features of ACF as the diseases end point in

each rat was found to be a more sensitive approach than their analyses by group. Using

this approach, growth features of ACF were able to predict the tumor stimulatory effect of

a high corn oil diet and inhibitory effect of ER.

The proliferative state of colonic epithelium has received a great deal of attention as a risk marker for colon carcinogenesis. Cell proliferation is a complex multi step process and is affected by several experimental variables and may be assessed by quantifying specific events occurring in one or more stages. Some of the common assessment techniques include quantification of cells in the S-phase (bromodeoxy uridine or ^{3}H-thymidine labelled cells), cells arrested in metaphase (mitotic index) or cells exhibiting PCNA. There are only a few studies that have compared the proliferative indices assessed as bromodeoxy uridine LI or PCNA LI (Yamada et al., 1992).

Our findings that the PCNA LI of the distal colon differs from that of the proximal colon in the LF groups is not surprising in view of the fact that one would expect distal colon to be different from proximal colon. What was worth noting is that the proximal colon did not respond to the dietary variables, fat and ER. This finding reasserted the notion that distal colon differs from proximal colon physiologically. Whether the distal colon normally has higher PCNA expression than the proximal colon is not well established. A study of site differences in cell proliferation within the human colon suggests that the rectal region does not differ from the sigmoidal region (O'Sullivan et al., 1992). In a recent study we observed in Sprague Dawley rats that the PCNA LI in the distal and proximal colons ranged between 6-14 regardless of the level of fat (corn oil) in their diet (unpublished observation). In another study conducted in female rats PCNA LI did not differ between the distal and proximal colons of animals fed a low corn oil diet (Lafave et al., 1994). An additional study conducted in mice (Caderni et al., 1991) reports that the LI (as assessed by [^{3}H] thymidine) and mitotic indices responded differently to

varying sources of dietary starch in the proximal region of the colon. A systematic

examination of the comparison of different indices of cell proliferation in different regions

of the azoxymethane treated F344 rat colon is lacking. It is generally believed and

reported in a few studies (Yamada et al., 1992) that the number of S-phase cells and

PCNA expressing cells in a crypt represents the proliferative state of the colon. However

it is important to note that this relationship is reported to be perturbed in the carcinogen

treated colonic crypts (Sutherland and Bird, 1994) . In a previous study the proliferative

status of colonic epithelium assessed by two different techniques did not lead to the same

conclusion (Roblee and Bird, 1989). The values for PCNA reported in previous studies

are considerably lower than the values reported in the present study (Lafave et al, 1994

and unpublished observation). The reason could be that F344 rats normally have higher

PCNA LI than Sprague Dawley rats. In addition, the rats in the present study were

injected with a colon carcinogen which in itself may affect PCNA LI. In the same context,

it can be suggested that the higher PCNA LI in the distal colon than in the proximal colon

noted in the present study is a reflection of the higher sensitivity of the distal colon to the

carcinogen AOM than the proximal colon.

It is apparent from the preceding discussion that there is sufficient evidence to

suggest that several experimental variables affect the proliferative state of the colonic

epithelium including variation in measurements among laboratories (Roe et al., 1996). A

recent study by Roe et al. (1996) assessed the reproducibility of the bromodeoxy uridine

labelling index quantification across four experimental laboratories. Their results suggest

that discrepancies exist from one site of analysis to another and that standardized training

in scoring techniques for all sites performing labelling index quantification may be required

to assure reproducibility across studies. To add to the existing complexities, only a limited

number of studies have evaluated the effect of ER or dietary fat on colonic cell

proliferation, and have failed to achieve conformity in various proliferative indices

(Albanes et al., 1990, Bird and Stamp, 1986, Klurfeld et al., 1987, Lok et al., 1988).

However, in two recent studies conducted in rats (Steinbach et al., 1993) and humans

(Steinbach et al., 1994), 20% ER significantly reduced the labelling indices in the distal

region of the colons. In the present study the ability of fat or ER in the LF groups to

affect proliferative indices in the distal region but not in the proximal region is worth

noting, and is congruent to the findings of Steinbach et al. (1993).

The preceding discussion suggests that further studies must be conducted to

delineate the effect of experimental variables on the cell cycle associated events and their

role in carcinogenesis. An increasing number of investigators are using PCNA LI as an

index of proliferative state and in the assessment of risk of colon carcinogenesis.

Therefore, it is important to establish the value of PCNA LI as a risk marker of colon

carcinogenesis.

The mechanism (s) by which dietary lipid (in this case corn oil) and ER mitigates

growth regulation in ACF or in colonic crypts remains open to a variety of suggestions.

The findings of the present study suggest that whatever the plausible mechanism (s) is/are,

certainly they are operating in a different manner across the regions of the colon. Since

we did not observe a positive correlation between the number of ACF and PCNA LI, it is

reasonable to suggest that growth regulation also differed between normal appearing

crypts and those that were developing into ACF.

In conclusion, the findings of the present study demonstrate that the growth features of ACF expressed as crypt multiplicity by rat or by group was the most sensitive measure for predicting the tumorigenic properties of fat and ER. In addition, it was demonstrated that a high corn oil diet stimulated the growth of ACF within four weeks and ER inhibited ACF growth by eight weeks. Dietary fat and energy may have affected the growth of ACF by two independent mechanisms. The distal colon responded differently than the proximal colon to dietary modulation as assessed by PCNA LI. Further research is needed to understand the mechanism (s) by which ER exerts a growth modulating effect on ACF and normal colonic epithelium.

7. MODULATION OF ABERRANT CRYPT FOCI BY DIETARY FAT AND ENERGY RESTRICTION: THE EFFECTS OF DELAYED INTERVENTION

7.1   Introduction

ER refers to a dietary regimen in which total energy has been reduced from carbohydrate or fat sources while increasing the vitamin and mineral content to ensure equal intakes between AL and ER groups. A plethora of physiological parameters may be altered by ER (Koizumi et al., 1987; Leakey et al.,1989; MacCay et al., 1939; Duffy et al., 1989; Semsei et al., 1989; Weindruch et al., 1988). Some of these effects include hormonal changes, alterations in circulating growth factors and their receptors and altered growth of neoplastic cells (Ruggeri et al., 1989a,1989b). These effects extend ER beyond the simple classification of energy deprivation or starvation and validate its proven ability to provide a "growth modulating environment" in which disease processes may be monitored. For several decades energy restriction has been associated with decreased incidence of spontaneous or chemically induced tumors in various animal tissues including chemically induced colonic tumors (Klurfeld et al., 1987; Kumar et al., 1990; Reddy et al., 1987). Such studies have convincingly demonstrated that ER is effective in inhibiting the development of colonic tumors when introduced at early stages of carcinogenesis. However, the mechanistic actions of ER which preclude the stepwise development of colon cancer remain elusive and is the focus of the present investigation. The concept that is exploited in the present

investigation is as follows. The genesis of cancer is a multi-step process involving sequential clonal selection and proliferation of initiated cells and their further development into identifiable focal lesions (Harris, 1991). The lesions, over time, exhibit advanced growth features and growth autonomy. Therefore, it can be hypothesized that preneoplastic lesions exhibiting varying growth features may respond differently to a growth modulating environment. In keeping with this concept whether mild ER is capable of retarding disease development, by affecting the early or intermediate preneoplastic stages of colon carcinogenesis, remains to be evaluated.

ACF were identified in carcinogen-treated rodent colons and purported to be putative preneoplastic lesions (Bird, 1987) with supporting evidence substantiating this hypothesis (McLellan and Bird, 1988a, McLellan and Bird, 1988b and McLellan et al., 1991). ACF with varying growth features are reported to occur in a rat colon after a single injection of a colon carcinogen (Bird,1987; McLellan and Bird, 1988a) presumably representing preneoplastic lesions at different developmental stages. Studies have supported the concept that ACF with a higher crypt multiplicity are more likely to develop into neoplastic lesions (Magnuson et al., 1993 and Zhang et al., 1992) suggesting ACF with increasing crypt multiplicity exhibit increasing potential to develop into cancer. The ACF model provides a framework in which the multi-step carcinogenic process subjected to a modulating environment such as ER may be analysed. This model also provides the unique opportunity to observe and analyse preneoplastic lesions that are very primal (ACF with 1-3 crypts/focus) or very

advanced (ACF with > 10 crypts/focus) in the same mucosal surface even at the later stages of carcinogenesis.

A culmination of the previously mentioned data substantiating ER as an effective tumor suppressant in colonic mucosa, the multi-step nature of cancer development and the ACF model provided the foundation for the present study. The basis of the present investigation was to assess the effects of delayed ER (20% less than the normal counterpart group), several weeks after carcinogen injection, on the intermediate stages of the colonic carcinogenic process in both high fat (HF 23% w/w) and low fat (LF 5% w/w) diets. ACF were employed as the biological end point of the disease, coupled with the analysis of colonic proliferative indices. Analysis of varying phenotypic features of ACF and cell kinetic responses at two time points were carried out to gain information into the preneoplastic events and possible mechanistic actions associated with ER which preclude colonic tumor development.

7.2   Materials and Methods

Animals

Eighty six male weanling F344 rats (Charles River Canada Inc., Montreal, Quebec, Canada) were used. Animals were housed in stainless steel wire cages with sawdust bedding with a 12 hour light-dark cycle. All animals were allowed free access to standard rodent laboratory chow and water (ad libitum) as an acclimatization measure for two weeks (14 days). Care of the animals was in accord with the guidelines of the Canadian Council on Animal Care.

Carcinogen

AOM, a known colon specific carcinogen, was dissolved in a fresh saline solution one hour prior to injection. After the acclimatization period, all animals were injected s.c. with AOM for two weeks (15mg/kg body weight once weekly).

Diets

Composition of the diets were based on the AIN-76A diet (see Chapter 4). All animals (n = 86) were fed an AIN-76A diet (5% corn oil w/w) after the final injection and continued on this diet ad libitum for 11 weeks at which time six animals were killed (0 TIME group) and the remaining eighty were then housed in single cages and randomly allocated to four groups (n = 20 per group). These groups received four dietary regimes, the AIN-76A HF, HFER, LF and LFER diets. Feeding protocols were followed as described in chapter 4. Body weights were determined every two weeks starting at the time of injection and weekly thereafter until the termination dates at 4 and 12 weeks.

Quantification of Aberrant Crypt Foci

All animals were killed by CO_2 asphyxiation. Two and a half hours prior to termination one half of the animals in each group received an injection of colchicine (1mg/kg body weight) (Sigma Chemical Co., St. Louis, MO) in order to arrest cells in metaphase. The remaining animals were not injected and were designated for PCNA analysis. Colons were removed and ACF characteristic determined according to the methods described in Chapter 3. Specifically, the total number and average crypt multiplicity were determined. In addition, the growth features of ACF in each group were further analysed by categorizing ACF into, small (1-3 crypts/focus), medium (4-6

crypts/focus) and large (>6 crypts/focus) classifications.

Tumor Identification

Identifiable tumors were defined using three criteria. The entire colonic mucosa was stained with methylene blue and examined under light microscopy. Microadenomas were defined as protruding microscopic lesions with an excessive number of crypts visualized microscopically at 40x. These lesions referred to as microadenomas, adenomas and adenocarcinomas exhibited dysplasia upon histological examination. Palpable lesions were fixed in 70% ethanol and embedded in paraffin wax. Sections were stained with haematoxylin and eosin and examined histologically and classified as adenomas or adenocarcinomas according to specific pathologic criteria described previously (Clinton et al., 1988).

Assessment of Proliferative Indices

Tissues from colchicine injected animals were stained with haematoxylin and eosin and evaluated for the presence of metaphase cells in complete longitudinal sections of colonic crypts. Metaphase cells were identified as darkly stained cauliflower shaped cells that were easily distinguished from normal colonic cells.

Tissues from animals not injected with colchicine were assessed for proliferating cell nuclear antigen (PCNA) as described in Chapter 3.

Statistical Analysis

SAS statistical software for microcomputers was used for all statistical analyses. Statistical analyses of: energy intake, organ and body weights, ACF data, proliferative indices and tumor multiplicity were performed by analysis of variance (ANOVA) and

Duncan's multiple range test. This data was further analysed using a two way ANOVA in order to determine the main effects of fat, ER and possible fat-ER interactions (fatxER). Tumor incidence was analysed by Fisher's exact tests. Correlations between ACF characteristics and food intake parameters were determined using Spearman's correlation coefficient. A P value ≤ 0.05 was considered significant in all statistical tests.

7.3   Results

Mean daily intake of diet and fat in grams/day and energy in kilojoules (KJ)/day were assessed among the four groups at week 4 and 12. In all measurements calculated, the ER groups consumed significantly less than their HF and LF counterparts (Table 7.1). Body and organ weights were monitored. Differences in body weights were evident at the third week of feeding with the restricted groups weighing significantly less than the HF and LF groups (Figure 7.1). At the tenth week of feeding significant differences were noted between all groups (with the HF weighing the most and the LFER group the least). This trend was maintained until termination of the study, with the exception of the LF and HFER groups attaining similar mass. The ER groups organ weights were generally less than their respective HF and LF counterparts.

Table 7.1 Mean daily intake values for male F344 rats fed one of four diets, HF, HFER, LF or LFER 4 and 12 weeks after dietary implementation [1].

		HF	HFER	LF	LFER
			MEAN DAILY INTAKE		
Diet g/day[2]	wk4	10.4 ± 0.2^b	8.4 ± 0.0^c	12.9 ± 0.2^a	10.2 ± 0.0^b
	wk12	11.1 ± 0.2^b	8.9 ± 0.0^d	13.0 ± 0.3^a	10.4 ± 0.0^c
KJ/day[3]	wk4	216.9 ± 3.8^a	175.1 ± 0.0^b	215.7 ± 4.2^a	170.9 ± 0.0^b
	wk12	213.5 ± 4.2^a	186.9 ± 0.0^c	218.2 ± 4.6^b	174.7 ± 0.0^d
Fat g/day[4]	wk4	2.4 ± 0.0^a	1.9 ± 0.0^b	0.6 ± 0.0^c	0.5 ± 0.0^d
	wk12	2.5 ± 0.0^a	2.1 ± 0.0^b	0.7 ± 0.0^c	0.5 ± 0.0^d

[1] Diets were implemented 11 weeks after injection.

[2,3,4] Values are mean $\pm$ S.E. (n= 10 rats/group)

Means in a row not sharing a common superscript are significantly different ($P \leq 0.05$, ANOVA and Duncan's multiple range test).

Fig. 7.1 Average body weights [mean + S.E.(bars)] of rats fed one of four diets 11

weeks after azoxymethane injection (n= 20 rats per group until 4 week, n=10 rats per

group 4 week until 12 week); HF, HFER, LF or LFER. Means not sharing a common

superscript at each week are significantly different ($P \leq 0.05$, ANOVA and Duncan's

multiple range test).
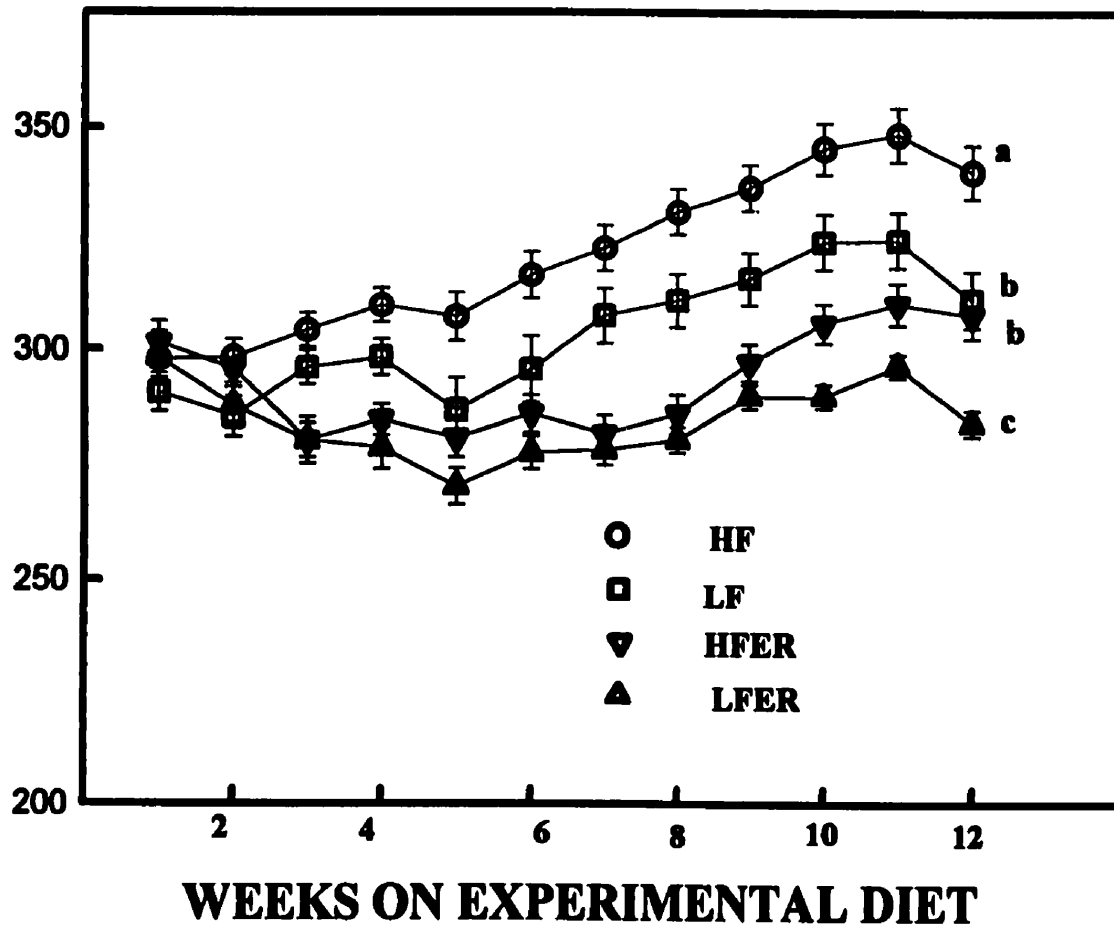
Table 7.2 Organ weights [1] and colon length [1] of male F344 rats fed one of four diets, HF, HFER, LF and LFER 12 weeks after dietary implementation [2]

ORGAN	WEIGHT IN GRAMS			
	HF	HFER	LF	LFER
COLON	1.50 ± 0.10^a	1.08 ± 0.04^b	$1.33\pm0.05^{a,b}$	1.07 ± 0.04^b
HEART	0.99 ± 0.02^a	0.84 ± 0.04^c	$0.93\pm0.02^{a,b}$	$0.87\pm0.03^{b,c}$
LIVER	8.07 ± 0.84^a	7.81 ± 0.17^a	8.34 ± 0.19^a	7.21 ± 0.16^a
KIDNEY	2.26 ± 0.04^a	2.03 ± 0.02^a	2.24 ± 0.10^a	2.11 ± 0.10^a
SPLEEN	0.65 ± 0.03^a	$0.60\pm0.02^{a,b}$	0.67 ± 0.03^a	0.54 ± 0.02^b
COLON LENGTH (CM)	18.4 ± 0.3^a	18.8 ± 0.2^a	18.2 ± 0.6^a	17.9 ± 0.2^a
COLON WEIGHT AS % OF BODY WEIGHT	0.44 ± 0.03^a	0.35 ± 0.01^c	$0.43\pm0.01^{a,b}$	$0.38\pm0.01^{b,c}$

[1] Values are mean$\pm$S.E. (n= 10 rats/group).
  Means in a row not sharing a common superscript are significantly different at $P \leq 0.05$ (ANOVA and Duncan's multiple range test).
[2] Diets were implemented 11 weeks after injection.

Colonic weight and their weight as a percentage of body weight were lower only in the HFER group compared with the HF group (Table 7.2).

ACF were present throughout the length of the fixed colon with the majority in the mid region (between 5 and 10 cm from the rectal end). A rapid increase in the total number of ACF in the HF group from the 0 TIME group ($P \leq 0.05$) was noted at week 4. However, there were no significant differences among the four experimental groups (Figure 7.2). At week 12, both the HF and LF had a significantly higher number of ACF than their corresponding ER groups (234.7 vs. 167.2 and 218.6 vs. 175.3 respectively).

The average crypt multiplicity (Figure 7.3) was not affected by ER or the level of fat in the diet at week 4. However, at week 12 the HFER group exhibited higher crypt multiplicity compared with the 0 TIME and LFER groups (3.5, 3.05, 3.02 respectively).

There were no apparent effects of ER on the number of small, medium and large ACF (see materials and methods for definition) at week 4 (Figure 7.4). The HF group had a significantly higher number of medium ACF than the 0 TIME group (53.4 vs. 32.2). At week 12 the number of small ACF were higher in the HF and LF groups compared to the ER groups (Figure 7.5). However, the difference between LF and LFER groups was not significant. The ER groups had lower numbers of medium ACF than their AL counterparts. The difference between the HF and HFER groups was not significant. A similar trend was observed for large ACF.

Fig. 7.2 Average number [mean + S.E.(bars)] of ACF in the whole colon/group at week 4 and week 12 in rats fed one of four diets 11 weeks after azoxymethane injection (n= 10 rats per group); HF, HFER, LF or LFER. O TIME represents rats terminated at the beginning of dietary implementation (n= 6 rats/group). Means not sharing a common superscript at each week are significantly different (P≤0.05, ANOVA and Duncan's multiple range test).
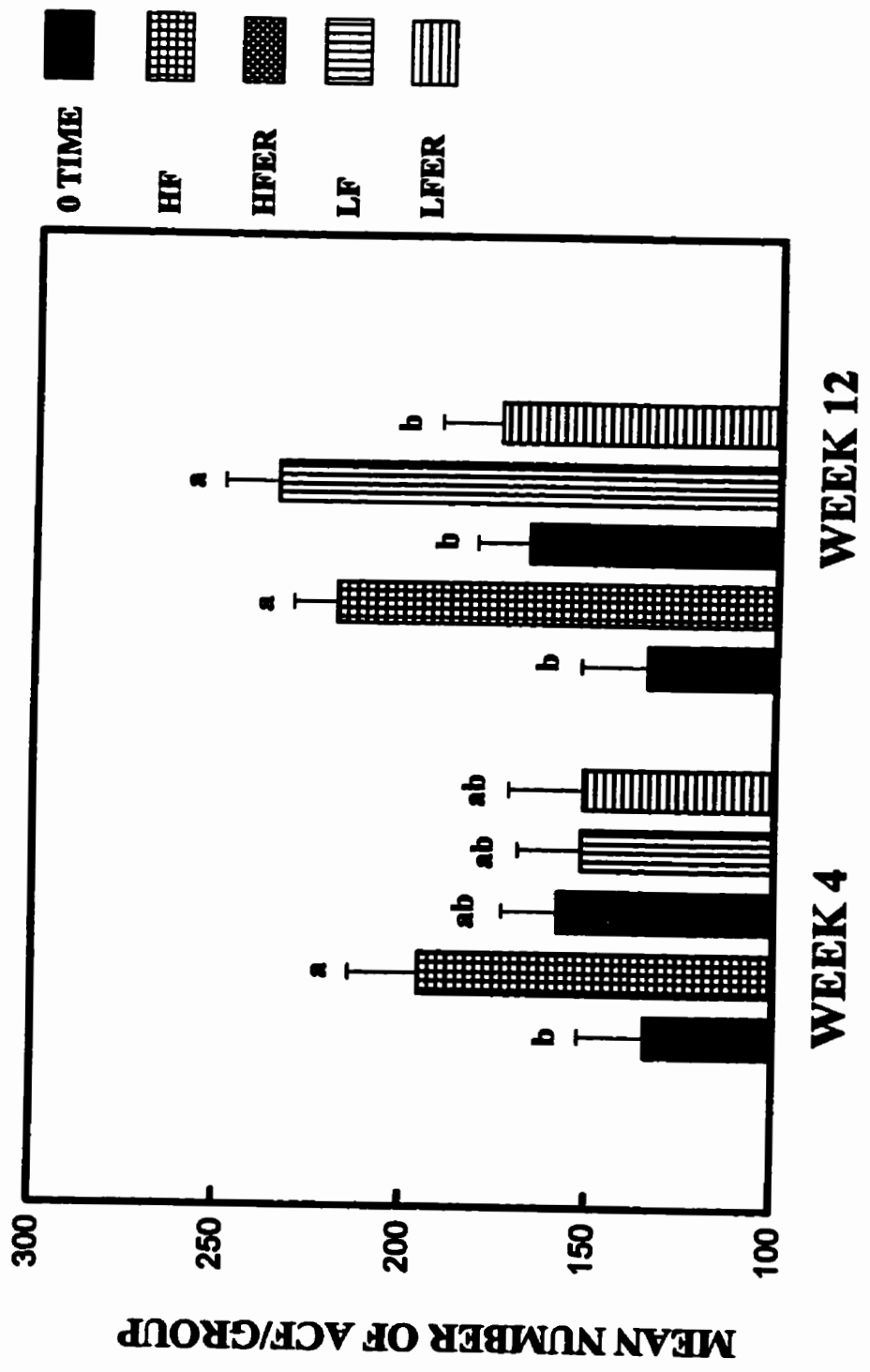
Fig. 7.3 Average number of crypt/focus [mean + S.E.(bars)]at week 4 and week 12

(n= 10 rats/group) in rats fed one of four diets 11 weeks after azoxymethane injection;

HF, HFER, LF or LFER. O TIME represents rats terminated at the beginning of

dietary implementation (n= 6 rats/group). Means not sharing a common superscript are

different ($P \leq 0.05$, ANOVA and Duncan's multiple range test).
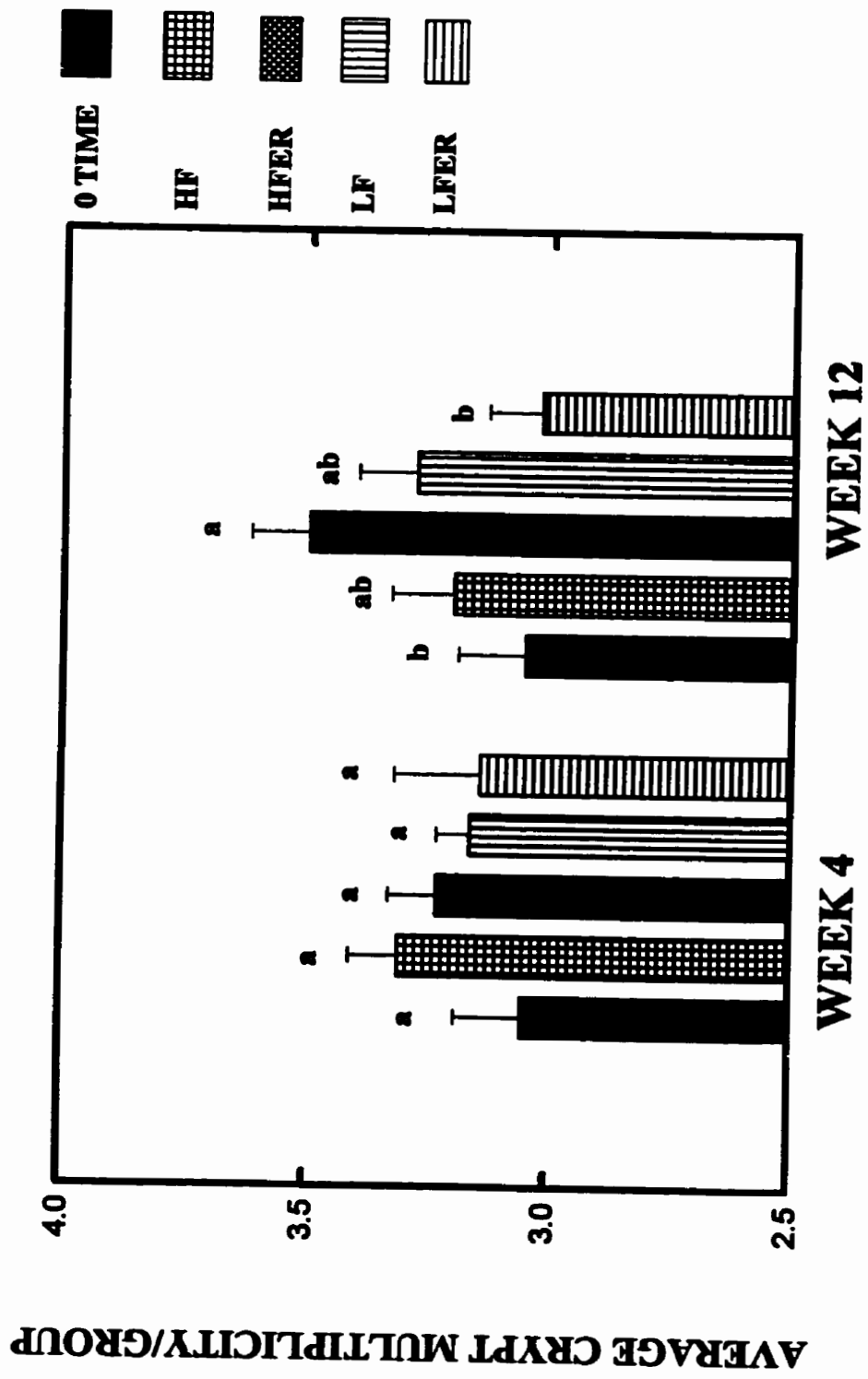
Fig. 7.4 Average number [mean + S.E.(bars)] of small (1-3 crypts/focus), medium

(4-6 crypts/focus) and large (>6 crypts/focus) ACF at week 4 in rats fed one of four

diets 11 weeks after azoxymethane injection (n = 10 rats per group); HF, HFER, LF

or LFER. O TIME represents rats terminated at the beginning of dietary

implementation (n= 6 rats/group). Means in each classification not sharing a common

superscript are significantly different (P≤0.05, ANOVA and Duncan's multiple range

test).
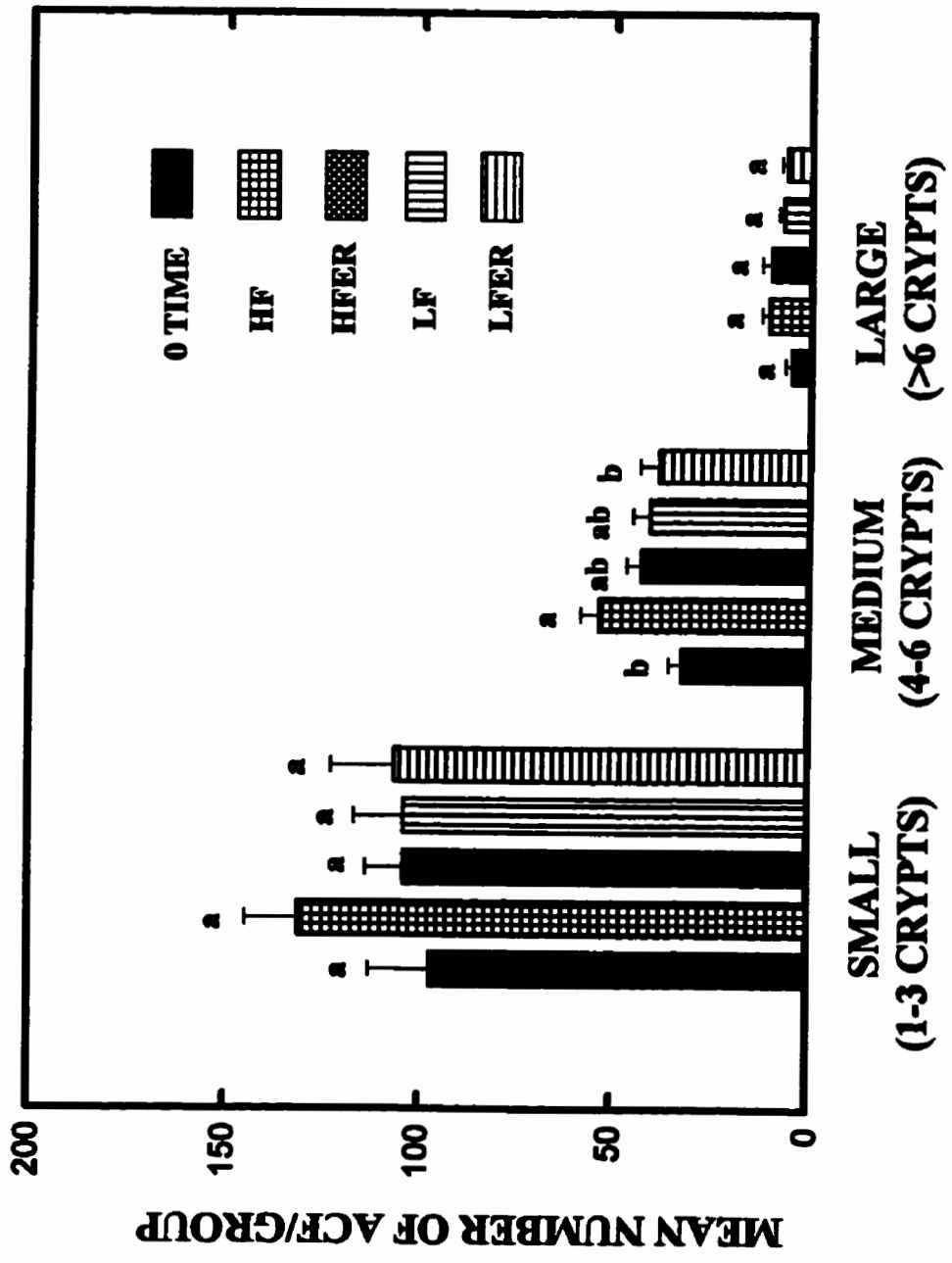
Fig.7.5 Average number [mean + S.E.(bars)] of small (1-3 crypts/focus), medium (4-6 crypts/focus) and large (>6 crypts/focus) ACF at week 12 in rats fed one of four diets 11 weeks after azoxymethane injection (n = 10 rats per group); HF, HFER, LF or LFER. O TIME represents rats terminated at the beginning of dietary implementation (n= 6 rats/group). Means in each classification not sharing a common superscript are significantly different (P≤0.05, ANOVA and Duncan's multiple range test).
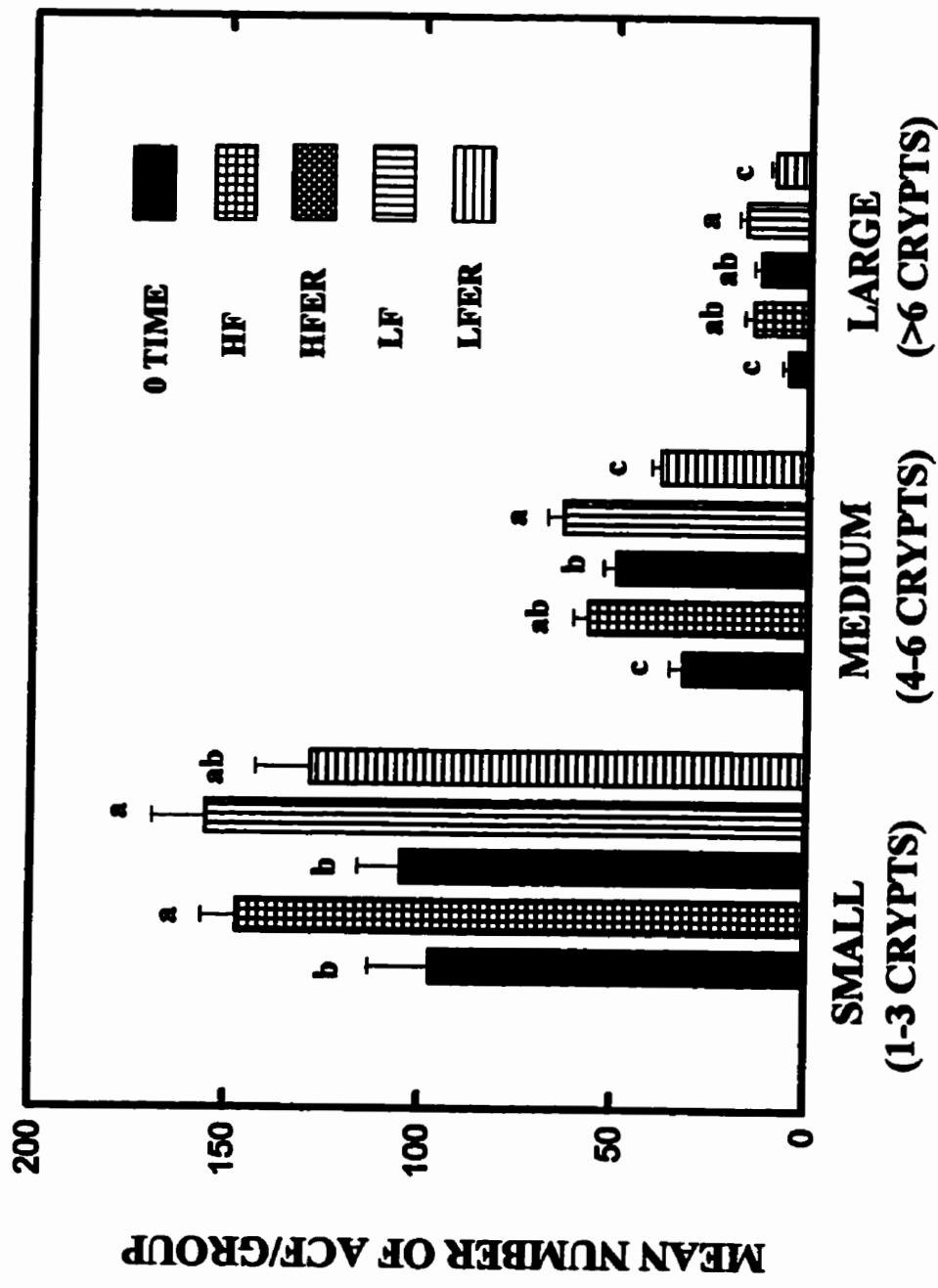
The main effects of fat and ER in modulating total ACF and ACF with different growth features are documented in Table 7.3. It is apparent that the main variable affecting the total number of ACF at week 12 was ER. The interaction between fat and ER was significant in affecting the average crypt multiplicity at week 12. Consequently, ER was identified as the significant variable affecting all three ACF multiplicity categories, small, medium and large. In addition, a fatxER interaction was identified as having a significant affect on medium ACF.

Correlation coefficients were determined between ACF characteristics and energy and fat intake on a per rat basis. At week 4 the only significant positive correlation existed between the number of medium ACF and the amount of fat consumed ($r=.34006$, $P=.0025$). At week 12, a positive correlation was observed between the total number of ACF and the average energy intake ($r=.46178$, $P=.0035$). Medium ACF positively correlated with both energy ($r=.64393$, $P=.0001$) and fat intakes ($r=.40644$, $P=.0113$) whereas large ACF correlated positively with energy intake ($r=.39394$, $P=.0144$).

The mitotic activity of the two different regions of colonic epithelium is documented in Table 7.4. In the proximal region a significant fat and ER interaction effect was noted at week 12 only. The MI of the distal region was affected by ER at week 4, however at week 12, no differences were noted among the groups.

The values for PCNA labelling indices are presented in Table 7.5. At week 4, ER groups had lower LI in the proximal section than their HF and LF counterparts however, these differences were not solely attributed to ER, but to a combined

Table 7.3 Two-way ANOVA *P* **values for male F344 rats fed one of four diets, HF, HFER, LF or LF 4 and 12 weeks after dietary implementation.**

	Mean no. ACF	Mean size ACF	Mean no. crypts/ focus	Mean no. Small ACF	Mean no. medium ACF	Mean no. large ACF	TBA[1]	TA[2]
WEEK 4								
FAT	0.1654	0.0259	0.3163	0.3381	0.0943	0.0437	0.0001	0.0015
ER	0.2851	0.0824	0.7082	0.3246	0.2108	0.7454	0.6390	0.6225
FATXER	0.3131	0.4327	0.8165	0.3275	0.2904	0.9953	0.4454	0.6225
WEEK 12								
FAT	0.5219	0.0349	0.1069	0.2652	0.3518	0.5947	0.3182	0.8699
ER	0.0004	0.7279	0.8625	0.0079	0.0001	0.0224	0.3286	0.0780
FATXER	0.7797	0.0020	0.0273	0.5526	0.0154	0.1703	0.7998	0.4151

[1]TBA = Tumors/tumor bearing animal in each group.

[2]TA = Tumors/animal in each group.

Table 7.4 Mitotic index[1] in distal and proximal sections of AOM treated colonic mucosa in rats fed one of four diets, HF, HFER, LF or LFER 4 and 12 weeks after dietary implementation [2].

Diet (n=5/ group)	Proximal Week 4	Proximal Week 12	Distal Week 4	Distal Week 12
HF	1.4 ± 0.2^a	$2.5\pm0.3^{a,b}$	$1.4\pm0.6^{a,b}$	2.3 ± 0.4^a
HFER	2.1 ± 0.3^a	$3.1\pm0.6^{a,b}$	0.3 ± 0.2^b	2.2 ± 0.6^a
LF	2.6 ± 0.6^a	3.7 ± 0.5^a	2.3 ± 0.5^a	3.1 ± 0.5^a
LFER	2.2 ± 0.4^a	2.4 ± 0.2^b	$1.0\pm0.3^{a,b}$	2.6 ± 0.4^a
Two-way ANOVA				
FAT	0.2482	0.4730	0.2066	0.2206
ER	0.9591	0.3031	0.0261	0.4819
FATxER	0.2991	0.0326	0.7927	0.6885

[1] Mitotic index = Mean number of metaphase cells/100 cells in each group.

[2] Dietary treatments were initiated 11 weeks after carcinogen injection.

Means in a row not sharing a common superscript are significantly different ($P \leq 0.05$, ANOVA, and Duncan's multiple range test).

significant fat and fatxER interaction. The distal LI were lower in the LFER group in relation to the LF group with no differences between the HF and HFER groups. Similar to the results obtained for the proximal section, the distal LI were affected by ER, fat and a fatxER interaction. PCNA LI at both time points proved to be paradoxical in nature. The HF group exhibited greater values than either LF or LFER groups with the exception of the proximal section LI. At week 12, decreased LI for the LF group in relation to the HF group was noted. Distal LI did not reveal any differences between the HF, LF and their respective ER groups, however the HF groups exhibited greater LI values than both LF groups. Fat, ER and a fatxER interaction were all significant variables in altering the distal LI values. There were no correlations between the PCNA LI and the mitotic figure MI or between ACF multiplicity classifications and these proliferative indices.

The majority of the microadenomas, adenomas and adenocarcinomas were located in the proximal region of the fixed colon consequently where the majority of the ACF were also identified. At week 4, (Table 7.6) the only identifiable tumors were in the HF groups. By week 12 there were no discernable differences between any groups in tumor incidence or tumor multiplicity. There were no differences in the number of invasive lesions among the treatment groups, however notable trends existed. Invasive lesions and adenocarcinomas in the HF group were twice as numerous than those in the LF group and virtually non-existent in either ER groups, but these differences were not significant. A combined tumor incidence for the HF groups and the LF groups over the two time points (n= 40/group) revealed a total tumor incidence of 45% and 17.5%

Table 7.5 PCNA labelling index[1] in distal and proximal sections of AOM treated colonic mucosa in rats fed one of four diets HF, HFER, LF or LFER 4 and 12 weeks after dietary implementation[2].

Diet (n=5/ group)	Proximal Week 4	Proximal Week 12	Distal Week 4	Distal Week 12
HF	15.0 ± 1.3^b	22.8 ± 5.9^a	28.4 ± 1.3^b	27.3 ± 2.0^a
HFER	8.7 ± 1.3^c	$20.6\pm1.3^{a,b}$	26.6 ± 2.0^b	26.4 ± 2.2^a
LF	28.7 ± 0.9^a	10.3 ± 2.1^b	38.1 ± 1.5^a	10.1 ± 0.6^c
LFER	6.5 ± 1.3^c	$12.4\pm1.8^{a,b}$	19.9 ± 1.6^c	19.6 ± 1.1^b
Two-way ANOVA				
FAT	0.0016	0.0093	0.0369	0.0001
ER	0.0001	0.9879	0.0001	0.0483
FATxER	0.0001	0.5427	0.0002	0.0105

[1] Mean number of PCNA labelled cells/100 cells in each group.

[2] Dietary treatments were initiated 11 weeks after carcinogen injection.

Means in a row not sharing a common superscript are significantly different ($P \leq$ 0.05, ANOVA, and Duncan's multiple range test).

**Table 7.6** Azoxymethane-induced colon tumor incidence and tumor multiplicity in male F344 rats fed HF, HFER, LF and LFER diets 4 and 12 weeks after diet implementation[1].

Diet	No. Animals/ Group	Colon Tumor Incidence				Tumor Multiplicity	
		Total[2]	Micro-adenomas	Adenomas	Adeno-carcinoma	TBA[3]	TA[4]
WK 4							
HF	10	50ᵃ(5)	50ᵃ(5)	10ᵃ(1)	0	1.4±0.24ᵃ	0.70±0.26ᵃ
HFER	10	30ᵃ·ᵇ(3)	30ᵃ·ᵇ(3)	0ᵃ	0	1.67±0.67ᵃᵇ	0.50±0.31ᵃᵇ
WK 12							
HF	10	70ᵃ(7)	60ᵃ(6)	30ᵃ(3)	20ᵃ(2)	1.57±0.30ᵃ	1.10±0.31ᵃ
HFER	10	30ᵃ(3)	30ᵃ(3)	0ᵃ	0ᵃ	1.00±0.00ᵃ	0.30±0.15ᵃ
LF	10	40ᵃ(4)	40ᵃ(4)	10ᵃ(1)	10ᵃ(1)	2.00±0.71ᵃ	0.80±0.42ᵃ
LFER	10	30ᵃ(3)	30ᵃ(3)	10ᵃ(1)	0ᵃ	1.67±0.33ᵃ	0.50±0.27ᵃ

Combined week 4 and week 12

	No. of animals/ group	Total[1]Tumor Incidence		No. of animals/ group	Total[1]Tumor Incidence
HF+HFER	40	45(18)	HFER+LFER	40	40(16)
LF+LFER	40	18(7)	HF+LF	40	23(9)

[1]Dietary treatments were initiated 11 weeks after carcinogen injection.
Values in the same column not sharing a common superscript are significantly different ($P \leq 0.05$, Fisher's exact test for tumor incidence and ANOVA with Duncan's multiple range test for tumor multiplicity.
[2]Total tumor incidence represent total number of animals with microadenomas, adenomas and adenocarcinomas
(actual number in bracket).
[3]TBA = Tumors/tumor bearing animal in each group.
[4]TA = Tumors/animal in each group.

respectively. Combination of the HF, LF and HFER, LFER groups over the two time

points (n= 40/group) revealed a total tumor incidence of 40% and 22.5% respectively.

7.4 Discussion

A novel approach was employed to assess the effect of ER on the intermediate

preneoplastic stages of colon carcinogenesis. ACF exhibiting varying growth features

were used as the target intermediate lesions. These lesions were induced by AOM and

allowed to develop for 11 weeks prior to carrying out the intervention with ER or

dietary fat. At the early time point, week 4, 20% ER did not reduce the total number

or the multiplicity of ACF within the HF or LF groups. The only correlation observed

between ACF characteristics and mean daily intake parameters was a positive

correlation between the amount of fat consumed and the number of medium ACF.

This demonstrated that dietary fat was able to affect the growth of ACF, 11 weeks after

injection, in a short period of time. These findings also suggested that an increase in

dietary fat affects tissue growth characteristics more rapidly than ER.

The effects of ER appeared to be more subtle and required a longer duration in

order to exert measurable responses at the tissue level compared with an increase in

dietary fat. The ability of ER to significantly alter (modify) the development of

preneoplastic ACF was evident at week 12 only. The amount of calories

consumed/day, or ER, was identified as a significant variable responsible for altering

the characteristics of ACF and proliferative indices more predominantly at week 12

than at week 4. Total ACF were significantly reduced by ER in both the HF and LF groups. Interestingly, modifications in ACF crypt multiplicity observed in the HF and LF groups were paradoxical in nature. ER in the HF group appeared to reduce the population of small ACF, whereas ER in the LF group reduced the population of medium and large ACF. These results reflect possible disparities between the mechanistic, evolutionary or inhibitory actions of ER present in LF and HF diets during the induction and promotional stages of carcinogenesis. It has been suggested that energy and fat intake exert different effects on colonic proliferation stemming from different mechanisms affecting colon carcinogenesis (Steinbach et al., 1993). ER in the HFER environment appeared to oppress the induction of new ACF, with no effect on the population of more advanced medium or large ACF relative to the HF group. An alternate interpretation is that the HFER diet inhibited the induction and stimulated the development of existing small ACF into medium and large ACF, considering there were no differences in the number of medium and large ACF between the HFER and HF groups. ER in the LFER environment appeared to oppress the development of medium and large ACF, with no effect on the induction of small ACF relative to the LF group. However, the number of small ACF in the LF group increased by approximately 50% from week 4 to week 12, whereas the LFER exhibited a 20% increase. This suggests that the LFER diet may actually stagnate the induction of new ACF relative to the LF group over time.

When ACF multiplicity data was expressed as percentage of total ACF, there were no observable differences between any dietary group at either time point (data not

shown).

The authors did not anticipate the appearance of sizeable microadenomas or adenomas in the present investigation nor was it part of the main scope or purpose of the paper. Interpretation of the data on lesions defined as microadenomas, adenomas and adenocarcinomas must be carried out with caution due to the small number of animals (n= 10/group/time point) used in the study. However, the data may provide insight into future investigations examining the effects of ER on ACF and end tumor incidence. The increase in the level of fat propagated the appearance of microadenomas and adenomas in the HF and HFER groups accompanied by the complete absence of such identifiable lesions in either LF groups at week 4. The total energy intake between the HF and LF or the HFER and LFER groups did not differ whereas the calories from fat were significantly higher in the HF groups compared with the LF groups. This supports the hypothesis that calories from fat are more instrumental in accelerating tumor development than total calories.

Week 12 total tumor incidence was lower in the ER groups relative to HF and LF counterparts. Adenocarcinomas were evident only in the HF and LF groups with the HF group tending to exhibit a greater incidence than the LF group although the difference was not significant. Consequently the only identifiable tumors in the HFER group existed at the level of microadenomas (no palpable tumors), whereas the HF group and both LF and LFER groups exhibited adenoma formation. Perhaps ER within a HF environment significantly alters growth of lesions that are very primal (ACF with 1-3 crypts) or very advanced (identifiable adenomas) in nature with no

detectable effect on intermediate lesions. The combined data (HF with the HFER group and the LF with the LFER group) suggests that increased dietary fat employed in the later stages of tumor development enhances the propagation of neoplastic growth. The analysis of this preliminary tumor data provides the basic reasoning for further investigations examining the effects of ER on end tumor incidence employing a larger sample size in order to evaluate this proposal.

Increased colonic proliferative rate is currently being labelled as a "colon cancer risk marker" (Yamada et al., 1992). A few studies have attempted to determine the existing relationship between ER, fat and cell proliferation in colonic mucosa. These studies have failed to achieve conformity in various proliferative indices (Albanes et al., 1990, Bird and Stamp, 1986, Klurfeld et al., 1987, and Lok et al., 1988) and eluded to the lack of association between increased tumor yield and cell kinetics among high fat diets (Steinbach et al., 1993). Steinbach et al. (1993) reported that a high fat diet decreased cell proliferation in the distal colon and increased proliferation in the proximal colon compared with a low fat diet after 34 weeks of feeding, with no differences occurring at the earlier time points. Lok et al. (1988) also demonstrated that a semi-synthetic diet, which is slightly higher in fat, reduced colo-rectal proliferation compared with lab chow fed rats. However, in both studies ER significantly reduced colonic proliferation. Klurfeld et al. (1987) demonstrated the ability of a diet containing three times the amount of fat but 40% of total calories to significantly increase colonic proliferation. Culmination of these results suggests that colonic cell proliferation may not be consistently affected by the amount of calories or

fat in the diet and thus may possibly depend on the frequency and amount of carcinogen, the duration of dietary treatment and the stage of carcinogenesis at which the analyses are being conducted. Early stage responses of cell kinetics to dietary changes may not represent the changes exhibited at the later stages. The varying PCNA LI expression results at week 4 and 12 may have been the result of an adaptive response. By week 12 there was a trend for the HF groups to have greater PCNA LI than the LF groups which reflects the response of the colonic mucosa exposed to the varying dietary treatments over a longer period of time. The finding that the distal and proximal section of the colons responded differently to dietary treatments, as assessed by their proliferative status, must be evaluated in future studies in order to determine the importance of this finding in experimental and possibly human colon carcinogenesis.

The findings of the present investigation demonstrated that carcinogen treated colonic epithelium is in a dynamic state exhibiting fluctuations in cell kinetics and the population of ACF. The present investigation supports a role for ER in cancer prevention. ER significantly modulated the growth of preneoplastic ACF without the introduction of any exogenous chemicals in the organism. Long term tumor incidence studies which administered ER diets immediately after carcinogen injection have well established the suppressive effects of ER over a long period of time. This investigation has substantiated the ability of a mild level of ER (20%) to significantly alter the growth of intermediate preneoplastic lesions which were allowed to develop for 11 weeks (post carcinogen injection) prior to dietary intervention. Increased dietary fat

affected tissue growth characteristics more rapidly than ER whereas ER altered the development of ACF depending on the level of fat and duration of feeding. ER inhibited different populations of ACF in HF and LF diets suggesting possible disparities between the cellular and molecular events occurring in each environment. Such findings support the contention that fat and ER affect the genesis of colon cancer by different mechanisms that are unique to each environment. In addition, it was demonstrated that ACF exhibiting different growth features responded differently to growth modulation by fat or ER,a finding which is compatible with the multi-step nature of the carcinogenic process. Information derived from the investigation of the intermediate lesions and their ability to respond to a growth regulatory environment may be useful in the management of high risk individuals harbouring preneoplastic lesions in their colon by dietary or chemopreventive means. The ability of ER to inhibit the appearance of new ACF and retard the development of established ACF warrants further studies. Such investigations may provide insight into the mechanistic actions of ER at the cellular level and the efficacy of ER as a means for cancer prevention.

8. INVESTIGATION OF THE ABILITY OF DIETARY FAT AND ER TO MODULATE VARIOUS RAT COLONIC PRENEOPLASTIC AND NEOPLASTIC LESIONS PROPAGATE INITIALLY IN A HIGH FAT ENVIRONMENT

8.1 Introduction

It has been demonstrated previously that the amount of fat and energy in the diet exerts a measurable response on the development of ACF in the early (Chapter 6) and intermediate stages (Chapter 7) of colon carcinogenesis. Previous studies have demonstrated the ability of decreased dietary fat and energy restriction (ER) to significantly decrease end tumor incidence when introduced at the early stages and maintained throughout the disease process (Klurfeld et al. 1987, Kumar et al., 1990 and Reddy et al., 1987).

Carcinogenesis is a complex process and involves clonal selection and expansion of initiated cells. The tumor enhancing effect of a high fat diet can be exerted on all, or a selected populations of preneoplastic lesions. We have recently demonstrated, in male Sprague-Dawley rats, that the tumor enhancing effect of a high beef tallow diet was established quite early on, within 12 weeks of carcinogen and dietary treatment (Bird et al., 1996). It was speculated that once preneoplastic lesions reach a certain growth state, they were resistant to the growth modulating affects of diet. This study suggested that administration of a growth permissive environment (high fat), soon after carcinogen injection, allowed for the appearance of more advanced preneoplastic lesions than those

which grew in a less permissive environment (low fat). Conversely, a high fat diet may select lesions with a genotype equipped for a more rapid growth pattern than those selected by a low fat diet.

Chapter 6 explored the delayed effect of intervention by HF, HFER, LF and LFER diets in colons that were initially exposed to a low fat environment. However, this study was conducted utilizing a limited number of rats, insufficient for a long term tumor incidence study.

Based on these two previous studies it was reasoned that administration of a high fat diet soon after carcinogen administration may provide a more stringent model to assess the effect of ER on the stepwise progression of colon cancer and formed the basis of this study.

Therefore, the objective of the present investigation was to assess the effects of ER (20% less than the normal counterpart group) on; ACF of varying growth features; microadenomas; and tumors. The dietary interventions (HF, HFER, LF, LFER) were implemented 16 weeks after feeding a high fat diet. Specifically, analysis of varying phenotypic features of ACF at three time points were carried out to gain information into the preneoplastic events associated with ER which preclude colonic tumor development. Microadenoma and tumor incidence and multiplicity were employed as the biological end points of the disease. Proliferating cell nuclear antigen expression was also assessed.

8.2   Material and Methods

Animal care and diets:

The animals were housed in wire cages with sawdust bedding with a 12h light-dark cycle. Temperature and humidity were controlled at 22°C and 55% respectively. Animals were given free access to laboratory chow and water until initiation of the experiment and over the duration of carcinogen injection. All animals were cared for according to the guidelines of the Canadian Council on Animal care. The diets were based on a semi-synthetic AIN-76 standard diet with modifications as specified in Chapter 4 Table 4.1.

Carcinogen

AOM was prepared according to Chapter 6. Animals were given 3 sub cutaneous (s.c.) injections of AOM at a dose of 15mg/kg body weight.

Study Design:

A summary of the study protocol is detailed in Figure 8.1. One hundred and sixty five male weanling F344 rats (Charles Rivers) were used. When the average weight of the animals reached 100 grams, all animals were then injected with AOM once weekly for three weeks and were allowed free access to lab chow over the duration of the injection period. One week after the last injection, all animals were switched to the modified AIN-76A diet HF for 16 weeks. After 16 weeks of dietary treatment, 10 rats were killed by CO_2 asphyxiation and their colons were evaluated for ACF characteristics. The remaining animals were housed in single cages and randomly allocated 40 per group into 4 experimental groups. These groups were fed one of the HF, HFER, LF or LFER diets. Diets within each fat classification were isocaloric, therefore a reduction in energy (20%) was accomplished by feeding the ER groups 80% of the average daily intake of their respective HF and LF counterparts as described in Chapter 4.

Figure 8.1 Study Protocol for Chapter 8 (black arrows designate injections with AOM, white arrows indicated termination of rats). Animals were fed standard lab chow during injections, then placed on the HF diet one week after last injection. Animals were allowed free access to the HF diet for 16 weeks at which time 10 animals were killed. The remaining animals were placed in single cages, separated into four groups (n=40/group) and fed one of four diets HF, HFER, LF or LFER. After six weeks (22 weeks after injection) and 12 weeks (28 weeks after injection) of feeding 10 animals/group and 30 animals/group were killed respectively.

Long Term Tumor Study Protocol

HF   HF

HF   HFER

HF   LF

HF   LFER

1 wk 1 wk 1 wk   16 wks   △ 6 wks   △ 6 wks   △
                                    n=10 rat from   n=10/group   n=30/group
                                    HF group

                              28 wks
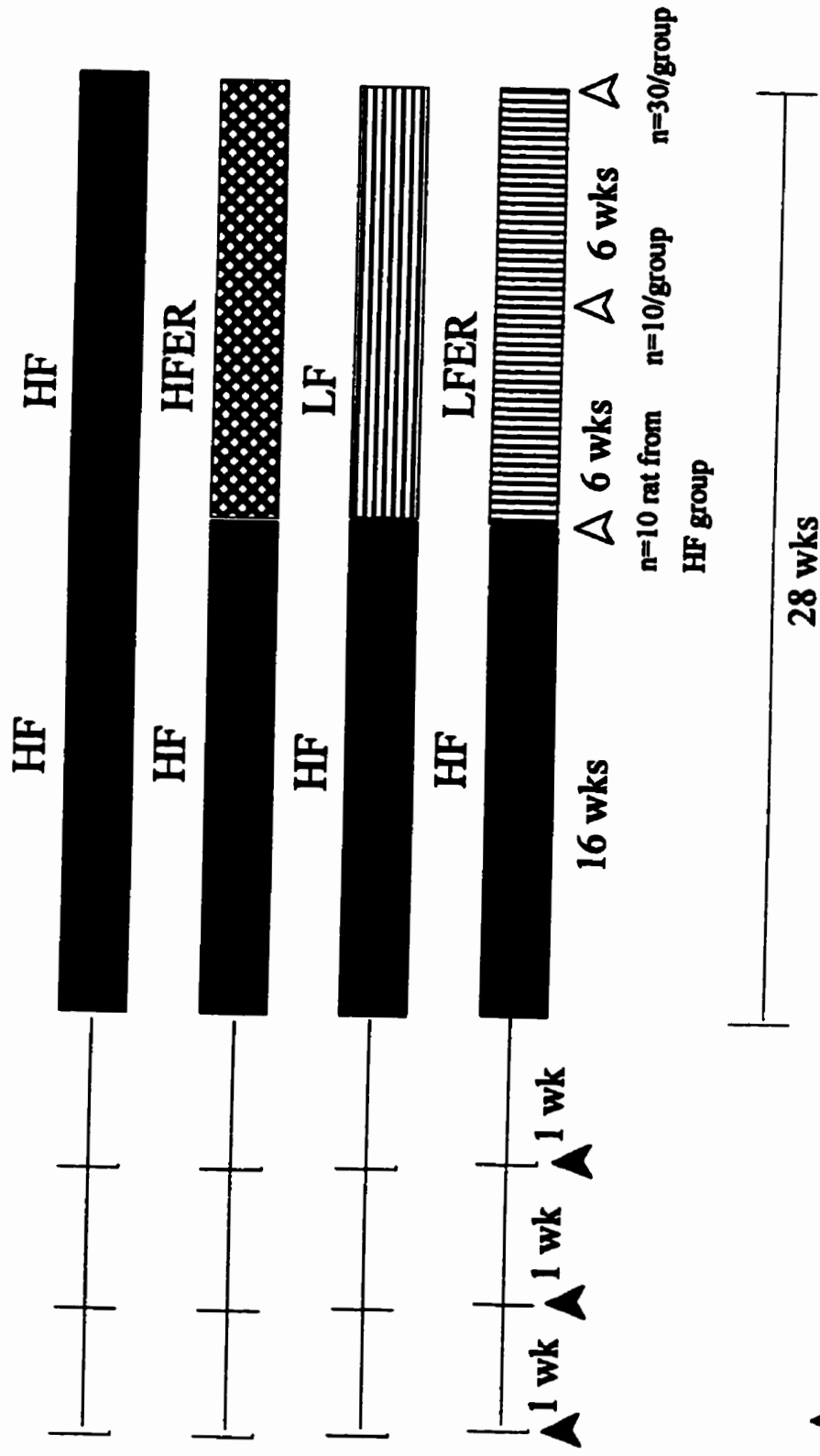
▲ = injection 15mg/kg AOM

△ = termination of animals

Initial and weekly body weights were recorded. After 6 weeks of feeding (22 weeks after injection) 10 rats per group were killed and their colons were analysed for ACF characteristics, microadenomas, tumors and proliferating cell nuclear antigen (PCNA) expression. The remaining animals (30 per group) were fed their respective diets for another six weeks (28 weeks after injection). Animals killed at this last time point were examined for macroscopic tumors and then further divided into 4 groups within each diet including; ten animals per group were designated for ACF analysis, (present chapter); ten per group for protein kinase C (PKC) analysis and epidermal growth factor tyrosine kinase activity (EGFR-TK) (see chapter 9); and the remaining were designated for RT-PCR (reverse transcriptase polymerase chain reaction) analysis (chapter 10).

Quantification of Aberrant Crypt Foci (ACF)

Animals were killed , colons removed, and ACF characteristics determined according to methods described in Chapter 3. Specifically, the characteristics analysed in this study include; total number of ACF; crypt multiplicity; number of small (1-3 crypt/focus), medium (4-6 crypts/focus), large (>6 crypts/focus) ACF; and location of ACF with varying crypt numbers along the length of the colon.

Tumor Identification and Quantification

Upon excision of the colon, all palpable lesions (tumor or microadenoma) were recorded with respect to their location and size (length and width). Non-palpable lesions were identified using light microscopy in the colons used for ACF analysis and were defined as protruding microscopic lesions with an excessive number of crypts visualized at X40. Lesions which were 1mm^2 and smaller were defined as microadenomas. Lesions

which were larger than 1mm² were defined as tumors. Specific parameters assessed were as follows: incidence (percentage of total animals with lesions); multiplicity calculated as average number of lesions per animal and average number of lesions per lesion-bearing animal; and burden (average of the total lesion area in each lesion-bearing rat in mm²). The total number of lesions in the group, irrespective of the number of rats in the group, was also assessed.

The parameters described above were assessed separately for tumors and microadenomas and for a combination of both tumors and microadenomas.

Assessment of Proliferating Cell Nuclear Antigen Expression

Proliferating cell nuclear antigen expression (PCNA) was examined in all groups at week 22 in the 70% ethanol fixed colonic tissue in the proximal and distal regions for all groups as described in Chapter 3.

Statistical Analysis

SAS statistical software for microcomputers was used for all statistical analyses. Body weights were analysed by repeated measures analysis of variance (ANOVA). ACF data, PCNA expression, tumor and microadenoma multiplicity were analysed by ANOVA and Duncan's multiple range test. This data was further analysed using a two way ANOVA in order to determine the main effects of fat, ER, and possible fat-ER interactions (fatxER). Tumor incidence was analysed by Chi square test. A P value $\leq$ 0.05 was considered significant in all statistical tests.

8.3 Results

ACF Characteristics

Base-line ACF characteristics were determined from the animals (n=10) terminated after sixteen weeks of feeding the HF diet, before the switch to the HFER, LF and LFER diets. The total number of ACF nearly doubled in all diet groups at the 22 week time point (6 weeks of feeding) compared to base-line (Table 8.1). The average crypt multiplicity was higher in the base line group, which reflects the lesser number of small ACF and a similar number of large ACF as compared to these characteristic in the 22 week diet groups. It is interesting to note that the increase in number of ACF at the 22 week time point in all diet groups was mainly due to the increased population of small ACF, rather than the large ACF. Within the diet groups at 22 weeks, there were no significant differences among the various ACF characteristics. However, there was a trend for the HFER and LFER groups to have lower numbers of total, small, and medium ACF and a higher number of large ACF than their respective HF and LF counterparts. Two-way ANOVA analysis of the ACF growth features at this time point did not reveal any significant main effects of fat or energy (data not shown).

After 12 weeks of feeding the four dietary regimens (28 weeks after injection) the total number of ACF increased in relation to the 22 week time point in all groups with the exception of LF (Table 8.2). The LF group exhibited a significantly lower number of total ACF than the HF and HFER groups. This suggests that switching from a HF to a low fat diet, rather than restricting energy (as in the HFER group), is more effective in reducing the total number of ACF. Consequently, fat was identified as the main variable affecting 12 week total number of ACF by two-way ANOVA analysis ($P \leq$ 0.0088, Table 8.3).

Table 8.1 ACF characteristics[1] of F344 rats at base line 16 weeks [2] and at 22 weeks [3] after injection.

DIET	TOTAL ACF/[1] COLON	AVERAGE [1] CRYPT MULT.	ACF [1] 1-3CRYPTS/ FOCUS	ACF [1] 4-6CRYPTS/ FOCUS	ACF [1] >6CRYPTS/ FOCUS
HF [3]	663.75 ± 71.54 [a]	2.41 ± 0.05 [b]	547.87 ± 59.38 [a]	102.25 ±13.21 [a]	13.63 ± 3.36 [a]
HFER [3]	587.60 ± 67.73 [a]	2.40 ± 0.06 [b]	477.60 ±54.43 [a]	94.20 ± 11.18 [ab]	15.20 ± 3.94 [a]
LF [3]	628.00 ±38.21 [a]	2.42 ± 0.05 [b]	515.18 ±34.67 [a]	100.73 ± 4.90 [a]	12.50 ± 1.95 [a]
LFER [3]	586.55 ±56.76 [a]	2.40 ± 0.08 [b]	487.73 ± 50.56[a]	87.82 ± 9.59 [ab]	15.27 ± 2.39 [a]
16 weeks [2]	351.89 ± 17.47[b]	2.76 ± 0.08 [a]	264.78 ±13.66 [b]	79.22 ± 8.22 [b]	12.43 ±2.25 [a]

[1] Values represent mean ± SEM (n=10/group)
  Means in a column not sharing a common superscript are significantly different (P≤0.05, ANOVA and Duncan's multiple range test).
[2] ACF characteristics 16 weeks after injection.
[3] ACF characteristic 6 weeks after switch to one of four diets HF, HFER,LF, LFER (22 weeks after injection).

The average crypt multiplicity was not affected by the amount of fat or energy in the diet. The LF group displayed significantly lower number of small and medium ACF than the HF and HFER groups respectively. It is interesting to note that the number of medium ACF decreased in LF group from the 22 to 28 week time points. Consequently, the number of medium ACF in the LF and LFER groups at 28 weeks was not significantly different than the base line number of medium ACF. Similarly, the LFER group exhibited a significantly lower number of medium and large ACF with than the HFER and HF groups respectively. Two-way ANOVA analysis revealed a significant fat effect on the population of small and medium ACF ($P \leq$ 0.0178 and 0.0057 respectively, Table 8.3).

Expression of PCNA

PCNA expression was assessed in distal and proximal sections at 22 weeks after injection (Table 8.4). The labelling index (LI) in the proximal region of the LF group was significantly lower in comparison to all other groups. There was a trend for the LI to be lower in the proximal end as compared to the distal end, with the exception of the HF group. ER appeared to decrease the LI in the proximal region of the HFER group and conversely increase the LI in the proximal region of the LFER group. The proliferative zone exhibited a trend to be higher in the proximal regions for all groups, with the exception of the LF group. ER did not affect the proliferative zone in the HFER group, however within the LF diets the LFER group demonstrated a significantly higher proximal proliferative zone than the LF group. Crypt height did not vary significantly from region to region, or from diet group to diet group once again with the exception of the LF group in the proximal region.

Tumor and Microadenoma Characteristics

Tumor and microadenoma parameters were analysed by combining the 22 and 28 week groups together (n= 33-44/group) and on week 28 alone (n=27-32/group). The location of tumors and microadenomas are summarized in Table 8.5. This data was not statistically analysed therefore the results must be assessed with caution. The majority of both types of lesions were found in the 4-12 cm of the colon in all groups which corresponds to the regions in which most of the ACF are located. Dietary fat and energy intake did not appear to affect the distribution of lesions along the length of the colon, however the LF group appeared to have a higher number of microadenomas in the 12-16 cm in relation to the other groups. In addition, the number of microadenomas in the LF 4-8cm region was higher than the rest of the groups, yet the number of tumors in this region was quite low. Colonic tumor incidence (Table 8.6) was not statistically different between the diet groups for the total (% of animals with tumors and/or microadenomas) tumor, or microadenoma and tumor incidence individually. The LF group exhibited the highest incidence of microadenomas, which was almost significant at $P = 0.051$, yet exhibited the lowest tumor incidence. There was a trend for the LF, LFER, and HFER groups to have higher total lesion incidence than the HF group. When the absolute total number of lesions is examined (Table 8.6) it was noted that the ER groups to harbour fewer total number of lesions than their respective HF and LF counterparts. The number of microadenomas in the LF group was more than double the number of microadenomas in the LFER group. It is interesting to note that although the LF group exhibited a higher number of microadenomas yet a lower number of tumors compared to the HF group.

Table 8.2 ACF characteristics[1] of F344 rats at base line 16 weeks [2] and at 28 weeks [3] after injection.

DIET	TOTAL ACF/ [1] COLON	AVERAGE [1] CRYPT MULT.	ACF [1] 1-3CRYPTS/ FOCUS	ACF [1] 4-6CRYPTS/ FOCUS	ACF [1] >6CRYPTS/ FOCUS
HF [3]	691.36 ± 4.35 [a]	2.54 ± 0.07 [a]	557.55 ± 42.24 [a]	107.55 ± 5.40 [ab]	24.18 ± 3.05 [a]
HFER [3]	676.83 ± 32.94 [a]	2.50 ± 0.05 [a]	542.08 ± 26.23 [a]	117.17 ± 8.35 [a]	20.00 ± 2.50 [ab]
LF [3]	537.91 ± 51.7 [b]	2.55 ± 0.06 [a]	429.55 ± 43.29 [b]	88.55 ± 7.76 [b]	18.91 ± 3.13 [ab]
LFER [3]	588.33 ± 44.56 [ab]	2.48 ± 0.05 [a]	478.33 ± 42.31 [ab]	94.33 ± 5.46 [b]	15.67 ± 1.41 [bc]
16 week [2]	351.89 ± 17.47 [c]	2.76 ± 0.08 [a]	264.78 ± 13.66 [c]	79.22 ±8.22 [b]	12.43 ± 2.25 [c]

[1]Values represent mean ± SEM (n=10/group)
 Means in a column not sharing a common superscript are significantly different (P≤0.05, ANOVA and Duncan's multiple range test).
[2]ACF characteristics 16 weeks after injection.
[3]ACF characteristics 12 weeks after switch to one of four diets HF, HFER,LF, LFER (28 weeks after injection).

Table 8.3 Two-way ANOVA *P* values of 28 week ACF characteristics

DIET	TOTAL ACF/ COLON	AVERAGE CRYPT MULT.	ACF 1-3CRYPTS/ FOCUS	ACF 4-6CRYPTS/ FOCUS	ACF >6CRYPTS/ FOCUS
FAT	0.0088	0.9501	0.0178	0.0057	0.0855
ENERGY	0.6845	0.3544	0.6695	0.2876	0.8639
FAT[1] X ENERGY	0.4632	0.7109	0.4120	0.7900	0.8639

[1]Test for a fat-energy interaction.

Table 8.4 Proliferating Cell Nuclear Antigen Expression (PCNA) indices in colonic distal and proximal sections of male F344 rats fed HF, HFER, LF and LFER diets killed 22 weeks after injection[1].

| DIET | Indices[2] | | |
	Labelling Index[3]	Proliferative Zone[4]	Crypt Height[5]
HF			
Distal	31.00±2.35^c	11.98±0.56^{bc}	28.37±0.37^{ab}
Proximal	46.75±2.69^a	16.73±0.95^a	29.55±1.22^{ab}
HFER			
Distal	35.00±2.16^{abc}	11.85±0.56^{bc}	27.55±0.90^{ab}
Proximal	31.50±6.59^{bc}	15.15±1.19^{ab}	30.47±1.56^a
LF			
Distal	35.73±3.69^{abc}	13.40±1.12^{abc}	29.47±1.03^{ab}
Proximal	16.42±2.13^d	10.50±0.39^c	21.65±0.83^c
LFER			
Distal	43.80±2.35^{ab}	14.04±1.15^{abc}	26.98±0.32^b
Proximal	39.25±6.28^{abc}	15.03±2.42^{ab}	26.82±1.14^b

[1] Dietary treatments were initiated 16 weeks after carcinogen injection.
[2] Values represent mean ± SEM (n=5/group)
  Means in a column not sharing a common superscript are significantly different ($P \leq 0.05$, ANOVA and Duncan's multiple range test).
[3] Represents the number of labelled cells/100 cells.
[4] Represents the highest labelled cell within a colonic crypt.
[5] Represents the number of cells along the length of a colonic crypt.

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Examination of lesion multiplicity parameters by grouping the 22 and 28 week

groups together did not reveal any significant differences for the number of lesions/animal,

number of lesions/lesion-bearing animal, or lesion burden (Table 8.8). However, when

lesion multiplicity is examined at 28 weeks alone (Table 8.9), the LFER group was

identified as having significantly lower numbers of tumors and/or microadenomas per

animal, tumors and/or microadenomas per lesion bearing animal, and a lower number of

microadenomas per microadenoma-bearing animal than the LF group. There were no

differences among the HF, HFER and LF groups. However, as reflected in the

incidence values, there was a trend for the HFER and LF groups to exhibit higher

values in almost all lesion multiplicity and burden parameters (with the exception of the

LF tumor/animal and tumor/tumor bearing animal values) than the HF group.

Table 8.5 Distribution (as a percent of total) of combined 22 and 28 week colon tumors and microadenomas along the length of the colon in rats fed HF, HFER, LF and LFER diets [1].

DIET	Location	of	Tumors[2]	
	0-4cm	4-8 cm	8-12cm	12-16cm
Tumors and microadenomas[3]				
HF	13 (8)	50 (31)	25 (15)	12 (7)
HFER	23 (13)	35 (20)	33 (19)	9 (5)
LF	16 (10)	39 (25)	23 (15)	22 (14)
LFER	17 (8)	37 (17)	33 (15)	13 (6)
Tumors [3]				
HF	6 (2)	61 (22)	25 (9)	8 (3)
HFER	26.5 (9)	29.5 (10)	35 (12)	9 (3)
LF	26 (7)	33 (9)	30 (8)	11 (3)
LFER	21 (6)	38 (11)	34 (10)	7 (2)
Micro-adenomas [3]				
HF	24 (6)	36 (9)	24 (6)	16 (4)
HFER	17.5 (4)	43.5 (10)	30 (7)	9 (2)
LF	8 (3)	43 (16)	19 (7)	30 (11)
LFER	12 (2)	35 (6)	29 (5)	24 (4)

[1] Dietary treatments were initiated 16 weeks after carcinogen injection.
[2] Location from rectal end.
[3] Values are percentage of total lesions in each group. Values in parenthesis are the actual number of lesions in that region for each diet group.

Table 8.6 Colon tumor incidence in male F344 rats fed HF, HFER, LF and LFER diets and killed 22 and 28 weeks after injection.[1]

Group	No. of animals/ group	Colon tumor incidence (% of total animals/group)		
		Total[2]	Microadenomas[3]	Tumors[4]
HF	44	70[a](31)	37[a](17)	55[a](24)
HFER	37	73[a](27)	35[a](13)	57[a](21)
LF	40	80[a](32)	48[a](19)	52[a](21)
LFER	37	76[a](28)	30[a](11)	59[a](22)
Total number of lesions/group[5]				
HF	44	61	25	36
HFER	37	57	23	34
LF	40	64	37	27
LFER	37	46	17	29

[1]Dietary treatments were initiated 16 weeks after carcinogen injection.
Values in the same column not sharing a common superscript are significantly different ($P \leq 0.05$, Chi square test)
[2]Total tumor incidence represents the percentage of animals with microadenomas and tumors. The number in parenthesis represents the actual number of affected animals.
[3] Microadenomas represent microscopic lesions ≤ 1 mm^2.
[4]Tumors represent macroscopic exophytic lesions > 1 mm^2
[5]Represents total number of lesions in group irrespective on the number of animals/group.

Table 8.7. Colon tumor incidence in male F344 rats fed HF, HFER, LF and LFER diets and killed 28 weeks after injection [1].

Group	No. of animals/ group	Colon tumor incidence (% of total animals/group)		
		Total[2]	Microadenomas[3]	Tumors[4]
HF	32	75ᵃ(24)	40ᵃ(13)	56ᵃ(18)
HFER	32	69ᵃ(22)	32ᵃ(10)	56ᵃ(18)
LF	30	73ᵃ(22)	43ᵃ(13)	53ᵃ(16)
LFER	27	78ᵃ(21)	30ᵃ(8)	59ᵃ(16)

[1]Dietary treatments were initiated 16 weeks after carcinogen injection.
Values in the same column not sharing a common superscript are significantly different ($P \leq 0.05$, Chi square test)
[2]Total tumor incidence represents the percentage of animals with microadenomas and tumors.
The number in parenthesis represents the actual number of affected animals.
[3] Microadenomas represent microscopic lesions ≤ 1 mm^2.
[4]Tumors represent macroscopic exophytic lesions > 1 mm^2.

Table 8.8 Colon tumor and microadenoma multiplicity and size in male F344 rats fed HF, HFER, LF and LFER diets and killed 22 and 28 weeks after injection [1].

TUMOR AND MICROADENOMA PARAMETER	DIETS			
	HF	HFER	LF	LFER
Tumors and Microadenomas (MA)				
Tumor and MA per² animal	1.38±0.18 ª	1.54 ±0.22 ª	1.60± 0.24 ª	1.24 ± 0.21 ª
Tumor and MA per² lesion bearing animal	1.90 ± 0.18 ª	2.11± 0.22 ª	2.00±0.25 ª	1.64± 0.24 ª
Tumor and MA² burden	17.12±4.73ª	26.48±7.84 ª	20.91±5.62 ª	16.16±3.84 ª
Tumors				
Tumors per² animal	0.81 ± 0.12 ª	0.92± 0.18 ª	0.68 ± 0.12 ª	0.78 ± 0.13 ª
Tumors per tumor² bearing animal	1.42 ± 0.10 ª	1.62± 0.21 ª	1.29 ± 0.10 ª	1.32 ± 0.14 ª
Tumor burden²	21.21± 5.81 ª	30.33±7.68 ª	30.38± 7.64 ª	19.82 ±4.46 ª
Microadenomas				
MA per animal²	0.57 ± 0.13 ª	0.63 ± 0.15 ª	0.93 ± 0.21 ª	0.46± 0.15 ª
MA per MA bearing² animal	1.35 ± 0.21 ª	1.77±0.17 ª	1.95 ± 0.29 ª	1.55 ±0.31 ª
MA burden²	1.35 ± 0.15 ª	1.37± 0.15 ª	1.63 ± 0.25 ª	1.50 ±0.29 ª

[1] Dietary treatments were initiated 16 weeks after injection.
[2] Values represent the total lesions at 22 and 28 week and are presented as mean ± SEM (n=37-44/group). Means in a row not sharing a common superscript are significantly different ($P \leq 0.05$, ANOVA and Duncan's multiple range test).

Table 8.9 Colon tumor and microadenoma multiplicity and size in male F344 rats fed HF, HFER, LF and LFER diets and killed 28 weeks after injection [1].

TUMOR AND MICROADENOMA PARAMETER	DIETS			
	HF	HFER	LF	LFER
Tumors and Microadenomas (MA)				
Tumor and MA per[2] animal	1.31±0.19 a	1.34 ±0.23 a	1.47± 0.25 a	1.04 ± 0.15 b
Tumor and MA per[2] lesion bearing animal	1.75 ± 0.18 ab	1.95± 0.24 a	2.00±0.25 a	1.33± 0.13 b
Tumor and MA[2] burden	19.62±6.01 a	21.02±6.33 a	24.39±6.90 a	16.33±4.80 a
Tumors				
Tumors per[2] animal	0.78 ± 0.14 a	0.78± 0.17 a	0.73 ± 0.14 a	0.70 ± 0.14 a
Tumors per tumor[2] bearing animal	1.39 ± 0.12 a	1.47± 0.19 a	1.37 ± 0.13 a	1.19 ± 0.14 a
Tumor burden[2]	25.28± 7.53 a	26.35±7.73 a	32.31± 8.44 a	20.81 ±5.75 a
Microadenomas				
MA per animal[2]	0.53 ± 0.14 a	0.56 ± 0.16 a	0.80 ± 0.20 a	0.33± 0.11 a
MA per MA bearing[2] animal	1.31 ± 0.21 ab	1.80±0.20 a	1.85 ± 0.25 a	1.12 ±0.13 b
MA burden[2]	1.23 ± 0.17 a	1.45± 0.16 a	1.65 ± 0.26 a	1.25 ±0.16 a

[1] Diet treatments were initiated 16 weeks after injection.
[2] Values represent total lesions observed at 28 weeks and are presented as mean ± SEM (n=27-32/group). Means in a row not sharing a common superscript are significantly different ($P \leq 0.05$, ANOVA and Duncan's multiple range test).

8.4   Discussion

The main findings of the present study are two-fold. Firstly, switching to a diet lower in total energy (HFER), fat (LF) or lower in energy and fat (LFER) in the later stages of colon carcinogenesis did not significantly modulate the development of tumors (neoplastic lesions) which were initially exposed to a HF environment during the early stages. Secondly, dietary fat was identified as a more potent growth modulator of certain populations of ACF (preneoplastic lesions) than ER. Previous investigations have demonstrated the ability of HFER and LFER diets to significantly reduce the tumor incidence in relation to their HF and LF counterparts (Kumar et al., 1991; Reddy et al., 1987). However, these studies have used experimental protocols in which the animals were fed the HFER and LFER diets while receiving or immediately after injection of carcinogen. The present study employed a protocol in which the diet intervention was implemented roughly half way through the carcinogenic process. The findings that delayed intervention by different diets did not change the tumor outcome corroborated a recent investigation by Bird et al. (1996) in which a similar protocol, although a different type of fat was utilized. In that study it was demonstrated that feeding a low and high beef tallow diet during the later stages of colon carcinogenesis was ineffective in modulating the growth of tumors (Bird et al., 1996).

Over the past few years, evidence has emerged supporting that notion that ACF are colonic preneoplastic lesions (Bird, 1995; Jen et al., 1994; Pretlow et al., 1991, 1992). Such investigations have identified key features of ACF that have strengthened this notion including dysplasia and proliferative and genotypic atypia parallel to those

identified in colonic cancer (Bird, 1995). It has been proposed that ACF of a higher crypt multiplicity may have a greater state of autonomous growth than those lesions with fewer crypts/focus (Magnuson and Bird, 1994). In addition it has been proposed that a shorter time is required to reach an autonomous state in a HF group than those in the LF group (Bird et al., 1996). Once an ACF attains a certain growth autonomy, it may be less amenable to growth modulation by the nutritional environment imposed upon the animal.

One of the advantages of intervening the disease process several weeks after the administration of azoxymethane is that at the time of dietary initiation, the colons harbor a large population of preneoplastic lesions. These lesions represent a spectrum of preneoplastic states. Therefore, utilization of this approach allows for the growth modulating ability of a nutrient or diet to be assessed on various preneoplastic states. As expected ACF of varying growth features were present in the colons of animals at week 16. Based on the findings of our previous study (Bird et al., 1996) we expected that some of the preneoplastic lesions would have reached the state of an established phenotype, enabling them to resist modulation by diet. However, we also expected that if ER was indeed a potent modulator of tumor development it would also be able to retard or regress the growth of preneoplastic lesions which had not yet achieved the autonomous or established phenotype.

Switching from a HF diet to a LF or LFER diet appeared to impede the development of the small and medium ACF yet did not affect the number of large ACF. Interestingly, the effect of decreased fat on the large ACF was not significant.

This is also reflected in the lesser number of total ACF in the LF and LFER groups, with the decrease mainly due to the lesser number of small and medium ACF.   This also suggests that within the LF and LFER diets, the larger ACF had reached a higher state of autonomy than the smaller ACF, reflecting biological heterogeneity among ACF within the same colon.

Reducing the amount of energy in the HF and LF diets did not appear to significantly affect the development of ACF. It has been demonstrated previously that reducing the energy content in HF and LF diets was able to significantly affect the development of certain populations of ACF within each fat classification (Chapters 6 and 7). It was also established that dietary fat exerted its effect at an earlier time point than the reduction in energy. However, these studies have employed different experimental protocols in relation to the present study. In particular , the study in chapter 6 introduced the diets low in fat and energy immediately after injection and the study in chapter 7 introduced the diets 12 weeks after feeding a low fat diet. The inability of ER to significantly affect ACF development in the HF and LF diets in the present study substantiates the notion that the diet in the early stages is more crucial in determining the preneoplastic and neoplastic potential of the colon.

In the present study, the exposure of all diet groups to a HF diet initially appeared to provide a tumor promoting environment that persisted even after the switch to lesser amounts of fat and energy in the diet. Although not statistically significant, it is interesting to note that there was a trend for the microadenoma incidence to be higher and the tumor incidence to be lower in the LF group in relation as compared to the HF group.

These findings corroborate our previous investigation (Bird et al., 1996) in which the increased tumor incidence of the HF-LF group in relation to the HF-HF group was due mainly to the appearance of small adenomas in the HF-LF group. Incidentally, similar findings have been reported in humans in which the reoccurrence rate of polyps was higher in individuals consuming a LF, high fiber diet as compared to those consuming more fat and less fiber (Eyssen et al., 1994). Such findings suggest that feeding a LF diet may not impede the development of microadenomas but may affected their progression to becoming tumors.

Reducing the amount of energy in the HF and LF diets did not significantly affect any tumor incidence parameter. With respect to tumor multiplicity, the LFER diet exhibited significantly lower tumors and/or microadenomas per lesion bearing animal than the LF group in the animals killed at 28 weeks. However theses values were not different than the HF group. Although not significant, the microadenoma incidence was higher in the LF compared to the HF group suggesting that switching from a HF to a LF diet may provide a microadenoma stimulating environment which may in turn be mitigated by the addition of ER.

Distribution of the colonic tumors for all diet groups was very similar, with the exception of the location of microadenomas in the LF group. The appearance of several microadenomas in the 12-16cm region of the LF colons suggests that either the HF diet was inhibiting, or the LF diet was stimulating the conversion of preneoplastic lesions into new microadenomas. In addition, the number of microadenomas in the 4-8 cm of the LF group was the highest, yet the number of tumors in that region were the lowest.

Consequently the HF group exhibited the highest number of tumors in the 4-8cm region, yet very low tumors in the 12-16 region. This suggests that there was a regional effect of LF diets. This diet appeared to inhibit the conversion of old microadenomas into tumors in the 4-8 region, while enhancing the development of new microadenomas in the 12-16 region. This trend was also demonstrated in the previous study by Bird et al. (1996). Carbohydrates serve as the main source of energy in LF diets which may indicate a role for increased carbohydrate intake in enhanced growth potential of certain preneoplastic lesions.

Proliferative indices, as measured by PCNA expression, reflected similar trends exhibited in the ACF, tumor and microadenoma data. There were no dramatic decreases or increases in the indices as a result of changing the amount of fat or energy in the diet. Previous studies have demonstrated the ability of ER to significantly decrease cell proliferation parameters (Steinbach et al., 1993, chapter 7). The results in the present study suggest that changing the amount of fat or energy in the diet within this study protocol does not significancy affect cell proliferative parameters as assessed by PCNA expression. It is interesting to note the decreased LI, proliferative zone and crypt height within the LF proximal region in comparison to all other groups. This region also corresponds to the region where the majority of the microadenomas were located. Such an observation does not support the contention that increased cell proliferation represents an increased risk for cancer development (Cohen and Ellwien, 1991). Recently, the validity of cell proliferation as a major risk factor for cancer has come into question (Farber, 1995). The results in the present study supports the suggestion that until a

system in which cell proliferation is the only, or the major, variable under study its role as a risk factor should remain in the "realm of unproven speculation" (Farber, 1995).

The major concept that is established in the present study is that feeding a HF diet in the early stages of colon carcinogenesis exerts a prolonged sustainable neoplastic growth promotional effect even after the amount of fat and energy is changed during the later stages of carcinogenesis. Dietary fat was able to modulate the growth of small/medium ACF while ER was unable to affect any population of preneoplastic lesions which eludes to the possibility that fat and ER exert their effects independent of each other. This concept substantiates the importance of identifying early, detectible biomarkers which are indicative of the presence or absence of disease in order to embark upon an effective and preventative diet/regime. The next step would be to determine whether or not energy restriction employed at the early stages would be effective in modulating ACF and microadenoma/tumor incidence after switching to a non-energy restricted environment in the later stages.

9. EGFR-TK, PKC ACTIVITY AND PKC ISOFORM EXPRESSION IN COLONIC MUCOSA AND TUMORS IN MALE F344 RATS

9.1 Introduction

Several investigations have supported the contention that tyrosine kinases, which catalyse phosphorylation of tyrosine residues in proteins, play an integral role in the regulation of cell proliferation, differentiation and transformation (Hunter and Cooper, 1985;Yarden and Ullrich, 1988). The family of PKC isozymes, which catalyse phosphorylation of serine/threonine residues of proteins, have also been substantiated as integral members of the regulation of cell growth and transformation (de Vente et al., 1995; Dlugosz and Yuspa, 1993; Guillem et al., 1987; McGarrity and Pfeiffer, 1994). Convergence of these pathways has been postulated at the membrane level, in particular with the interaction between the tyrosine kinase associated with the epidermal growth factor receptor (EGFR-TK) and its "cross talk" with membrane associated PKC (Ullrich and Schlessinger, 1990).

Activity of both PKC and EGFR-TK have been shown to be significantly affected by carcinogen treatment (Craven and DeRubertis, 1992b; Malecka-Panas et al., 1996). However, it is not known how the activity of these enzymes may respond to changes in dietary fat and energy implemented at an intermediate stage of colon carcinogenesis (refer to Chapter 8 study protocol). In addition, the activity of these enzymes have not been assessed in conjunction with each other.

In the present study, it was of interest to analyse the activity of these enzymes in

colonic mucosa and tumors in rats fed diets varying in fat and energy content during the last 12 weeks prior to their termination (28 weeks after injection of AOM). The specific objectives of the study were to determine the effects of ER in high (23%) and low (5%) fat diets on; a) PKC and EGFR-TK activity in colonic mucosa and tumors; and b) the expression of PKC isoforms in colonic mucosa and tumors.

9.2 Materials and Methods

Animals, Diets, Carcinogen, Study protocol

Animals used were those designated for PKC and EGFR-TK in chapter 8. Specific details pertaining to the type of animals, diets, carcinogen and study protocol are detailed in Chapter 8 materials and methods.

Extraction, Separation of Colonic Cytosol and Membrane Fractions for PKC activity and Western Blotting

The colonic mucosa designated for PKC activity and western blotting procedures were prepared according to the protocol in Chapter 3. Tumors from each diet group were pooled together to amount to 0.3 gm (approximately 3-5 tumors) and processed according to the protocol for separation for the PKC assay as described in Chapter 4.

PKC Assay

The PKC assay was conducted on the membrane and cytosol fractions on colonic mucosa and tumors utilizing the Amersham kit as described in Chapter 3.

Extraction, Separation of Membrane Fractions for EGFR-TK activity

The initial stages of separation to obtain the cytosolic fraction and membranous

pellet are described in Chapter 3. After these steps, the method of membrane preparation for the EGFR-TK assay followed that of Malecka-Panas et al. (1996). The membranous pellet was then resuspended in 3 mls of resuspension buffer containing 10mM Hepes, 150 mM NaCl, 1mM MgCl$_2$, 1 mM PMSF, 1 mM Na$_3$VO$_4$, 1 mM 1, 10-phenanthroline, 10 µg/ml Leupeptin, 1µl/ml Aprotinin, 0.1% Triton-X 100 and 0.5% Nonidet P-40. The membrane fractions were then frozen at -80°C.

Immunoprecipitation of EGFR

Immunoprecipitation was conducted according to the method described by Malecka-Panas et al. (1996). Immunoprecipitation of the EGFR from the membranous fraction (isolated by the method described in the previous section) was required in order to assess the activity of the tyrosine kinase associated with this receptor. Initially, Pansorbin cells (CalBiochem, La Jolla, California) were centrifuged as 3000 x g for 10 minutes, then suspended in an equal volume of PBS (pH 7.2), containing 10% w/v β-mercaptoethanol and 3% w/v SDS. The cells were then boiled for 30 minutes and centrifuged for 10min at 3000 x g. The pelleted cells were then washed in PBS to remove the β-mercaptoethanol and SDS. The cells were then used for the immunoprecipitation procedure.

In order to activate the receptor, 200 µg of membrane fraction protein was incubated with 6 µM ATP and 1x10^{-8} M of transforming growth factor-α (TGF-α) for 15 minutes on ice. and stopped with an equal volume of RIPA buffer (see appendix B) with 1 µl of polyclonal anti-EGFR sheep antibody (UBI, Lake Placid , NY). The fractions were then incubated for 2 hours at 4°C with the antibody.

After 2 hours, 35 µl of treated Pansorbin cells were added to each membrane

fraction and incubated for another 2 hours. The fractions were then microfuged at 10,000 x g for 10 minutes. The pellet was resuspended in 60 µl of the resuspension buffer and split into two fractions (30 µl each, in order to have a duplicate reading in the assay) and kept on ice until the time of the assay.

EGFR-TK assay

The EGFR-TK assay was conducted on the same day and immediately after immunoprecipitation. Each 30 µl sample was incubated with 20 µl of cocktail containing 2.5 µl IBS (1 M Tris pH 7.8, 1M $MgCl_2$, 2 mM Na_3VO_4), 3 µl ATP (60 µM) and 12.5 µl polymer of glutamic acid:tyrosine (4:1) (Sigma) (20 µg/ 5 µl) and 2 µl γP^{32}-ATP (.5 µCi, 3000Ci/mmol). Incubation was conducted for 10 minutes at room temperature. The reaction was then stopped by spotting the whole reaction (50 µl) onto filter paper (P 81, Whatman) and then placed immediately into 5 ml of 10% tricholoro-acetic acid (TCA) and 0.2% sodium pyrophosphate (NaPP). Samples were then washed once and incubated in vials containing 5 mls of TCA/NaPP solution overnight at 4°C. The next day, samples were washed three times with the TCA/NaPP, placed in 5 mls of Cyto-Scint and read for one minute on a L-6000 Beckman Scintillation Counter. The blank was prepared by conducting the assay on 35 µl of treated Pansorbin cells. The assay was also conducted on the membranous fractions without stimulation by TGF-α. Results were calculated as the average between the duplicates minus the blank and expressed as pmol Pi transferred/100 µg protein.

Protein Analysis

Protein in colonic mucosa and tumor membrane and cytosol fractions was

determined according to the method described in Chapter 3.

Detection of PKC Isoforms expression by Western Blotting

PKC α andγ isoforms were detected in colonic mucosal cytosolic and membranous fractions. PKC α, γ, δ, ϵ, ζ, were detected in colonic tumors. The methodologies and calculations are described in Chapter 3.

Statistical Analysis

Statistical analysis of PKC activity, PKC isoform expression and EGFR-TK activity was carried out using Analysis of Variance (ANOVA) in conjunction with Duncan's Multiple Range Test. This data was further analysed using a two-way ANOVA in order to determine the main effects of fat, ER, and possible fatxER interactions. Correlations between PKC activity and PKC isoform expression were performed using Pearson's and Spearman's correlation coefficients. All test were conducted using the SAS statistical software package for microcomputers (SAS Institute Inc., Cary, NC). A P value ≤ 0.05 was considered significant.

9.3 Results

PKC activity

Colonic and tumor PKC activity is detailed in table 9.1. In general, membranous PKC activity was higher than the cytosolic activity in all diet groups. However this difference was significant only for the tumors and the LFER groups. As in the previous studies (Chapters 4 and 5) the variability within diet groups was quite high for both the cytsolic and membrane fractions. Although not significant, it is interesting to note that the ER groups tended to have higher activity in the membrane fraction, and lower activity in

Table 9.1 Colonic mucosa and tumor Protein kinase C activity[1] in male F344 rats fed one of four diets HF, HFER, LF, LFER and killed 28 weeks after carcinogen injection.[2]

DIET		Total PKC Activity[3]	Cytosolic PKC Activity	Membranous PKC Activity
HF	mucosa	289.22 ±74.21ᵃ	48.86 ± 29.09 ᵃ	242.30 ±54.96ᵃᵇ
	tumor	1026.44	43.64	982.8
HFER	mucosa	279.51 ±117.14ᵃ	11.88 ±8.55 ᵃ	267.63 ±110.72 ᵃᵇ
	tumor	200.23	57.95	142.28
LF	mucosa	216.18 ±69.26 ᵃ	44.93 ±20.47 ᵃ	171.29 ±67.91 ᵃᵇ
	tumor	40.10	12.41	27.69
LFER	mucosa	366.55 ±114.24 ᵃ	25.08 ±8.89 ᵃ	341.47 ±107.29 ᵇ*
	tumor	689.15	17.87	671.28
Average of All Tumors		489.01 ±226.18 ᵃ	33.00 ±10.76 ᵃ	456.01 ± 224.67 ᵃ*

[1] Activity expressed as pmol Pi/min/mg protein, values are means±SEM. Means in a column sharing a common superscript are not significantly different ($P \leq$ 0.05 ANOVA with Duncan's multiple range test).

[2] Diet treatments were initiated 16 weeks after injection.

[3] Total PKC represents cytosolic + membranous activity

*Denotes significant difference between the membranous and cytosolic fraction ($P \leq$ 0.05 ANOVA with Duncan's multiple range test).

the cytosolic fraction as compared to their respective HF and LF counterparts. Tumor PKC activity was also higher in the membrane fractions in relation to the cytosol for all diet groups. In comparison to the mucosa, the tumors exhibited higher membrane associated activity in relation to the HF and LFER groups, and lower cytosolic activity than the HF, LF and LFER groups. The average size of tumors used in the assay was the lowest in the LF group (22 mm^2) compared to HF, HFER and LFER groups (38 mm^2, 30 mm^2, 48 mm^2 respectively). Therefore, the decrease in PKC activity in the LF tumor group may reflect possible effects of tumor size on PKC activity. Total PKC activity, which represents the membrane and the cytosolic activity , was not significantly different among the groups. Two-way ANOVA did not reveal any significant main effects of fat, ER, or a fatxER interaction (data not shown).

PKC isoform Expression in Colonic Mucosa and Tumors

PKC α and γ expression are detailed in Figures 9.1 and 9.2 respectively. Immunoblotting was conducted on equal amounts of protein in the cytosol and membrane fractions from all diet groups and tumors that were used in the PKC assay. Both isoforms gave strong protein band signals at approximately 80 kDa. Figure 9.3 details the loading gel pattern for a particular diet. Five samples from a particular diet were run on the same gel with both the cytosolic and membranous fractions (ten samples per gel). All groups exhibited strong signals in each isoform except for the HFER cytosolic fraction which did not exhibit any expression of PKC γ. There was no consistent observable pattern of diet effect on either isoform. The only significant difference observed was the expression of cytosolic PKC α in the LF group being higher than the LFER and HFER groups. There

Figure 9.1 Average normalized areas of protein kinase C α protein bands in colonic

mucosa cytosolic and membrane fractions in rats fed one of four diets, HF, HFER, LF,

LFER and killed 28 weeks after injection (diets were implemented 16 weeks after

injection). Values are means±SEM(bars).

Average normalized area (y-axis): 1.0, 0.8, 0.6, 0.4, 0.2, 0.0

Categories: CYTOSOL, MEMBRANE

Legend:
- Tumor
- HF
- HFER
- LF
- LFER
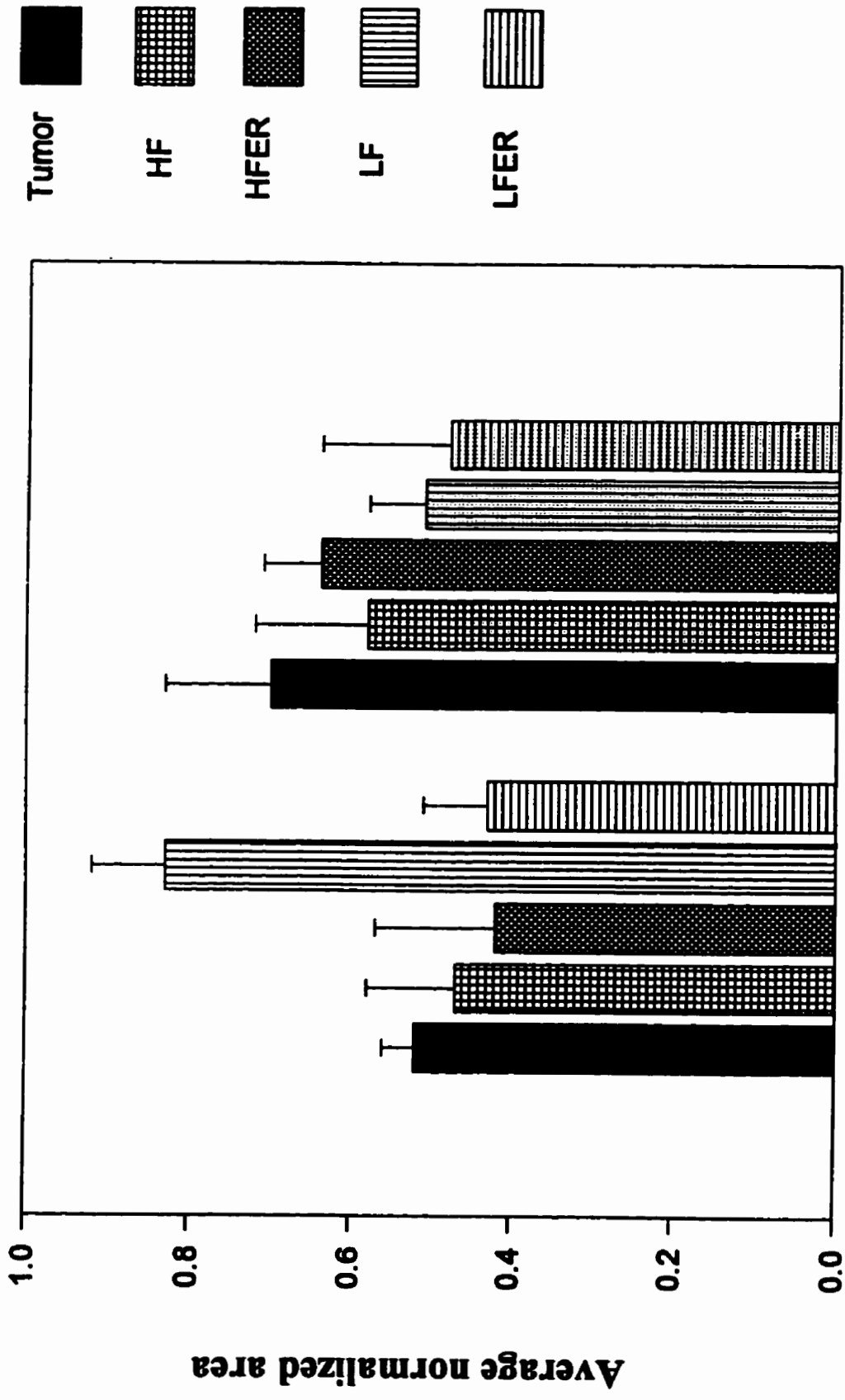
Figure 9.2 Average normalized areas of protein kinase C γ protein bands in colonic
mucosa cytosolic and membrane fractions in rats fed one of four diets, HF, HFER, LF,
LFER and killed 28 weeks after injection (diets were implemented 16 weeks after
injection). Values are means±SEM(bars).

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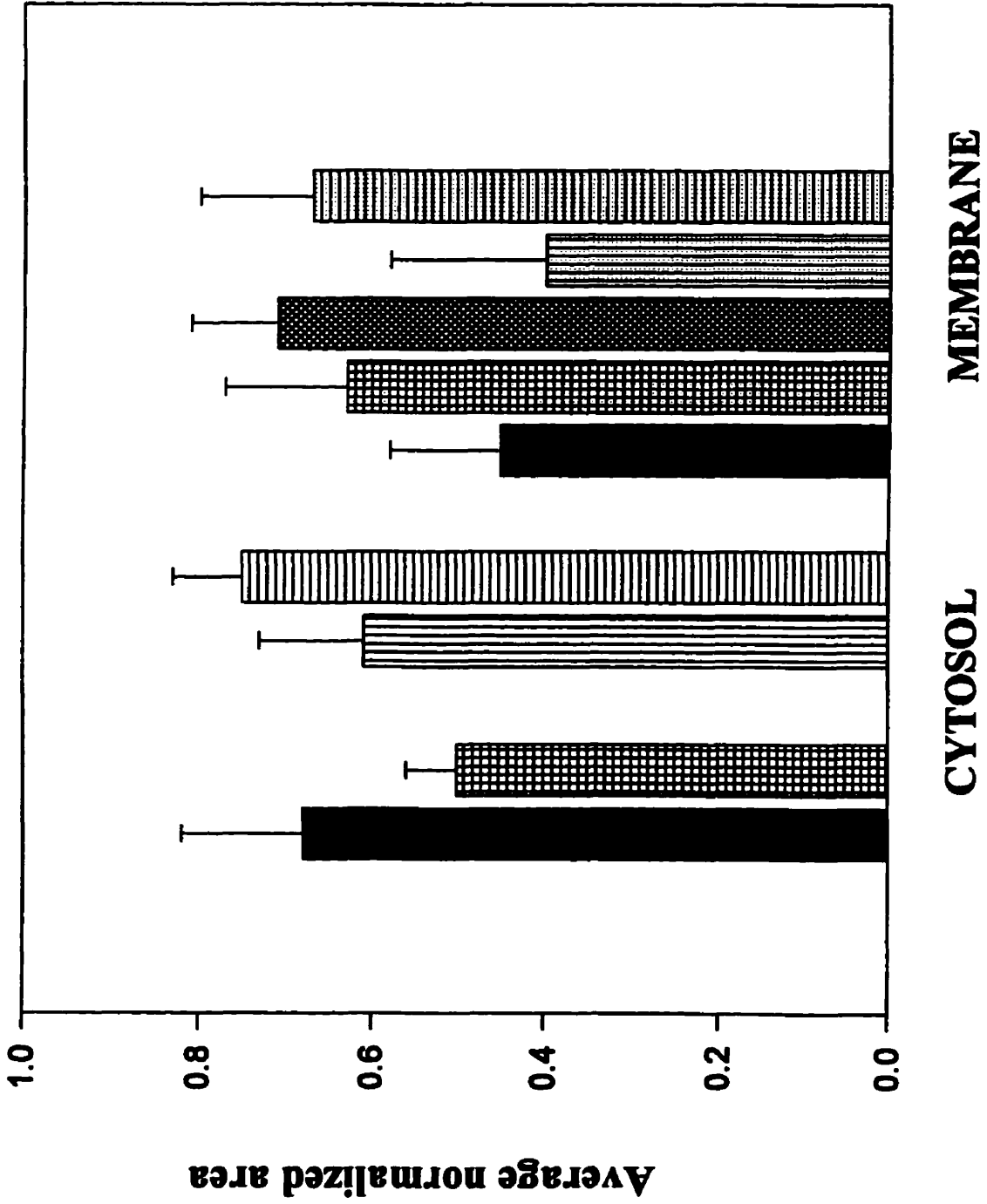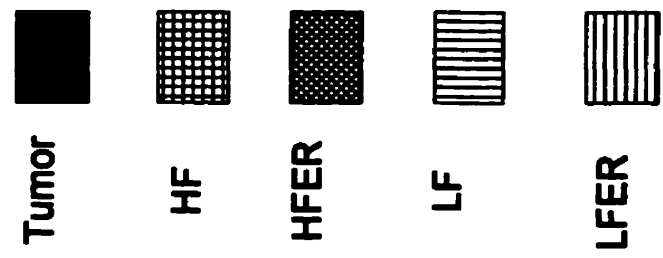
Figure 9.3 Membranous and cytosolic PKC γ in HFER and PKC α in HF colonic

mucosa from rats killed 28 weeks after injection (diets were implemented 16 weeks

after injection). Protein bands were detected by enhanced chemiluminescence and

developed on Kodak X-OMATAR film. Positive controls were HeLa and Jurkat cell

lines for PKC α and γ respectively (Transduction Laboratories, Lexington, Kentucky).

PKC Isoforms as Detected by Western Blotting Techniques

PKC γ
HFER

PKC α
HF

80 kDa

Positive control

Membrane   Cytosol

80 kDa

Positive control
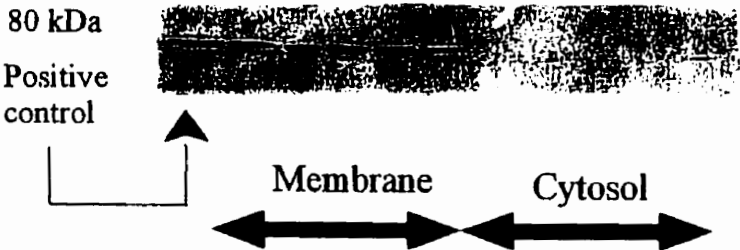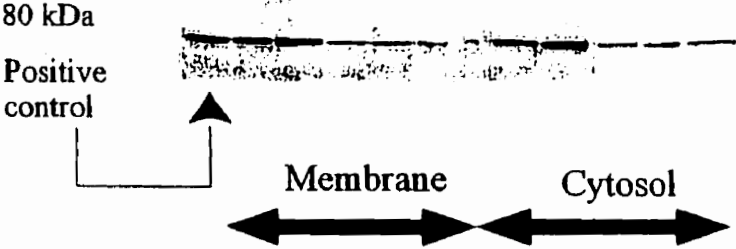
Membrane   Cytosol

were no significant correlations between any PKC activity and PKC isoform parameter (data not shown).

Tumor cytosolic and membranous isoform expression is detailed in Figures 9.4 and 9.5 respectively. Approximately 2-4 tumors were pooled together from each of the four diet groups with the cytosolic and membranous fractions run on the same gel (eight lanes per gel with the cytosolic and membranous fraction from each of the four diets). All isoforms gave signals at approximately 80 kDa. This data was not statistically analysed, due to the small number of samples and the inability to compare one isoform to another. Therefore, the results must be assessed with caution. There were no discernable patterns or effects of diet on any of the isoforms. However, it is interesting to note the lack of PKC δ in the cytosolic fraction and the lack of PKC ζ in the membranous tumor fractions (Figure 9.6).

EGFR-TK activity

EGFR-TK activity is detailed in Tables 9.2 and 9.3 and was measured with and without stimulation by TGF-α. Stimulated EGFR-TK activity was significantly higher in the HFER group in relation to all other groups. The range of activity for all groups was from 4.85 - 9.51 pmol Pi incorporated/100 µg protein which are comparable to a previous investigation analysing EGFR-TK in AOM treated colonic mucosa (Malecka-Panas et al., 1996). There was a trend for the activity to be higher in the HF groups as reflected by the significant fat effect ($P = 0.0454$, Table 9.3). ER was also identified as a significant variable affecting stimulated EGFR-TK ($P = 0.0253$) which is reflected by the higher activity in ER groups. Although not significant, stimulated average tumor activity (as an

Figure 9.4 Normalized areas of protein kinase C α, γ, ϵ, and ζ protein bands in cytosolic

fractions of colonic tumors in rats fed one of four diets, HF, HFER, LF ,LFER and killed

28 weeks after injection (diets were implemented 16 weeks after injection).

Normalized area

Legend: HF, HFER, LF, LFER

Categories: PKC α, PKC γ, PKC ε, PKC ζ
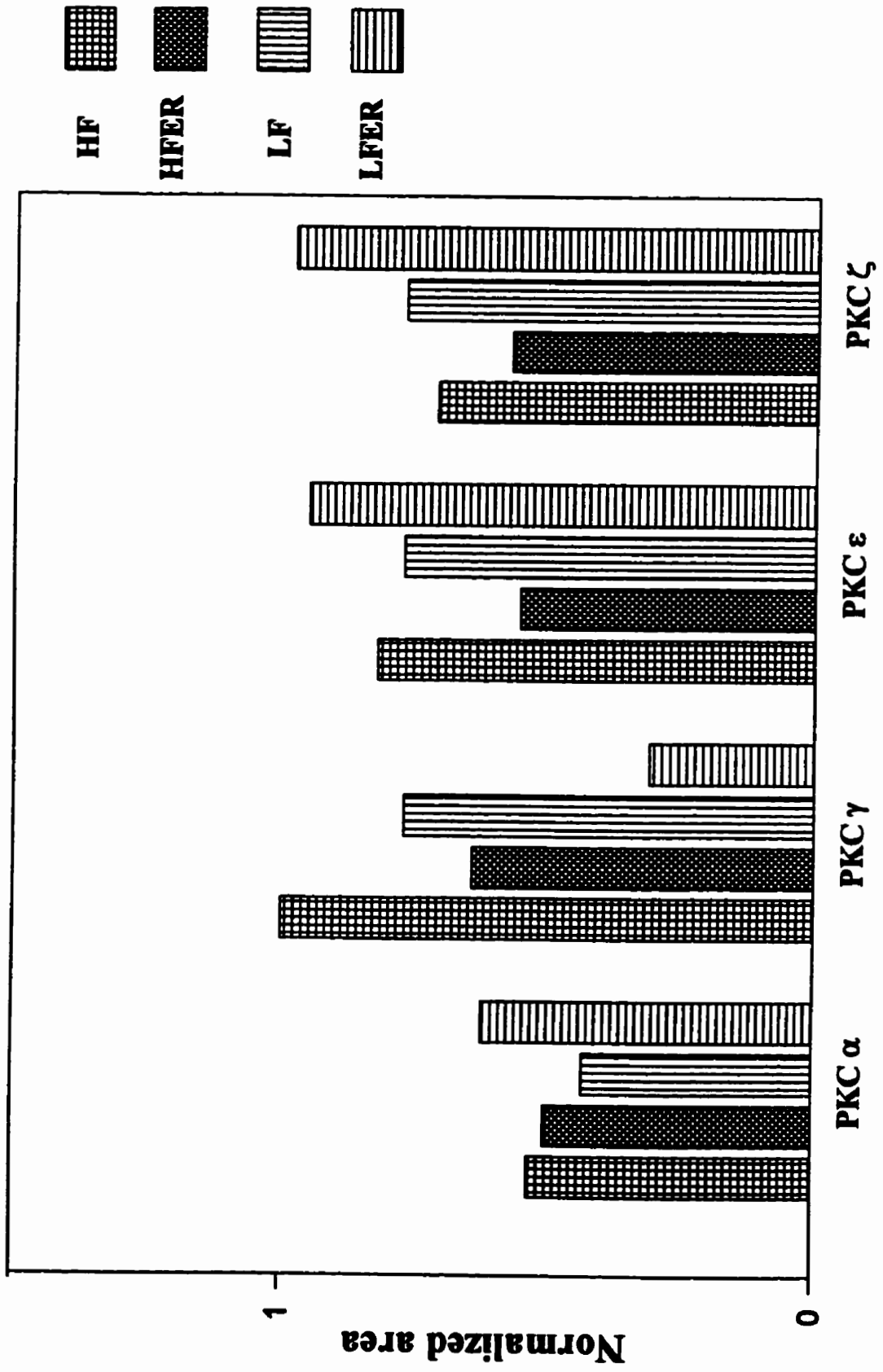
Figure 9.5 Normalized areas of protein kinase C α, γ, δ, and ϵ protein bands in membrane fractions of colonic tumors in rats fed one of four diets, HF, HFER, LF, LFER and killed 28 weeks after injection (diets were implemented 16 weeks after injection).
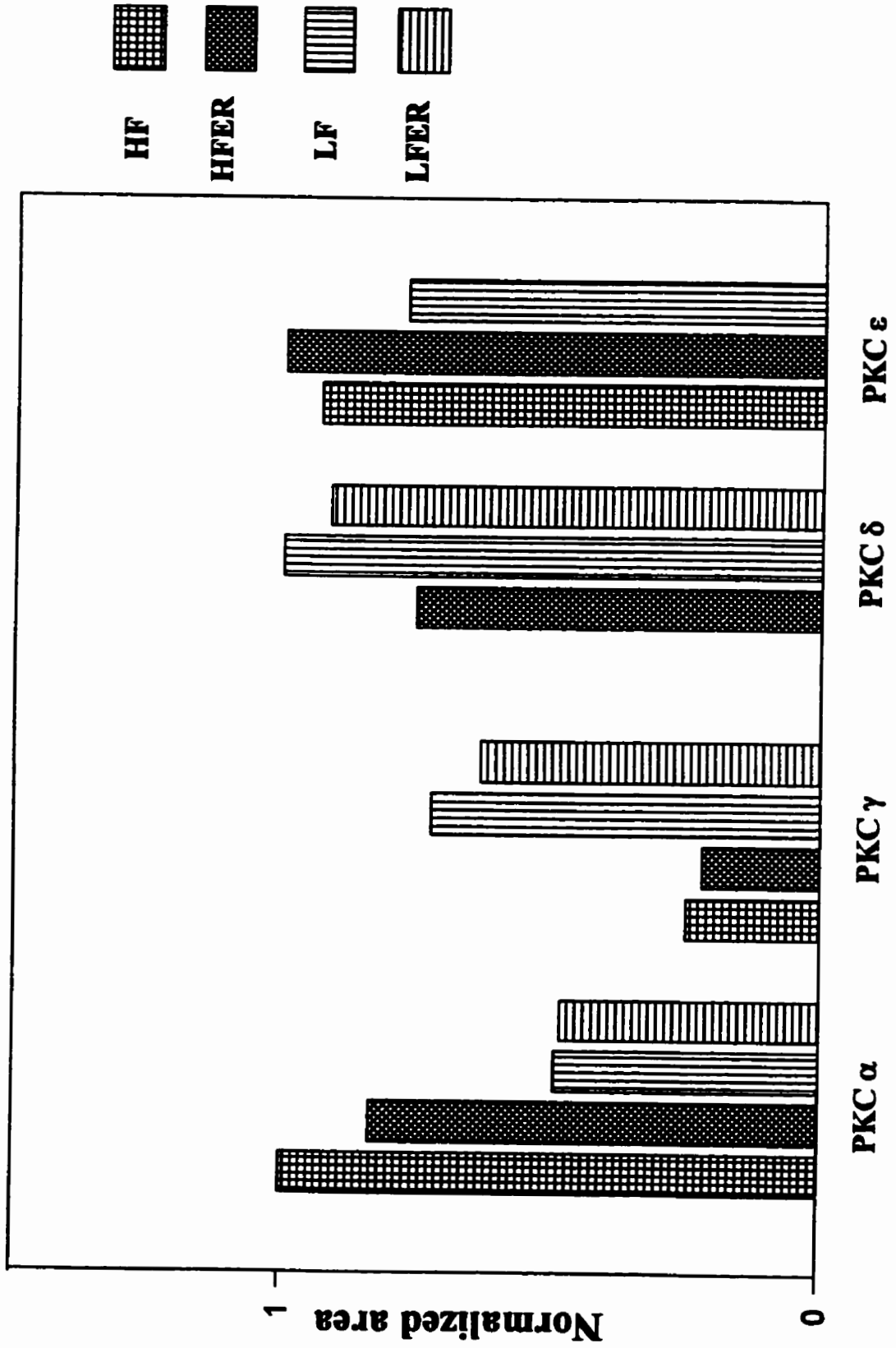
Figure 9.6 Membranous and cytosolic PKC isoforms α, δ, and ζ colonic tumors in rats fed one of four diets, HF, HFER, LF, LFER and killed 28 weeks after injection (diets were implemented 16 weeks after injection). Protein bands were detected by enhanced chemiluminescence and developed on Kodak X-OMATAR film. Positive controls were HeLa , mouse macrophage and MDCK cell lines for PKC α and δ and ζ respectively (Transduction Laboratories, Lexington, Kentucky).

PKC Isoforms in Tumors as Detected by Western Blotting Techniques

PKC α

80 kDa

Positive control

Membrane   Cytosol

PKC δ

80 kDa

Positive control

Membrane   Cytosol

PKC ζ

80 kDa

Positive control

Membrane   Cytosol
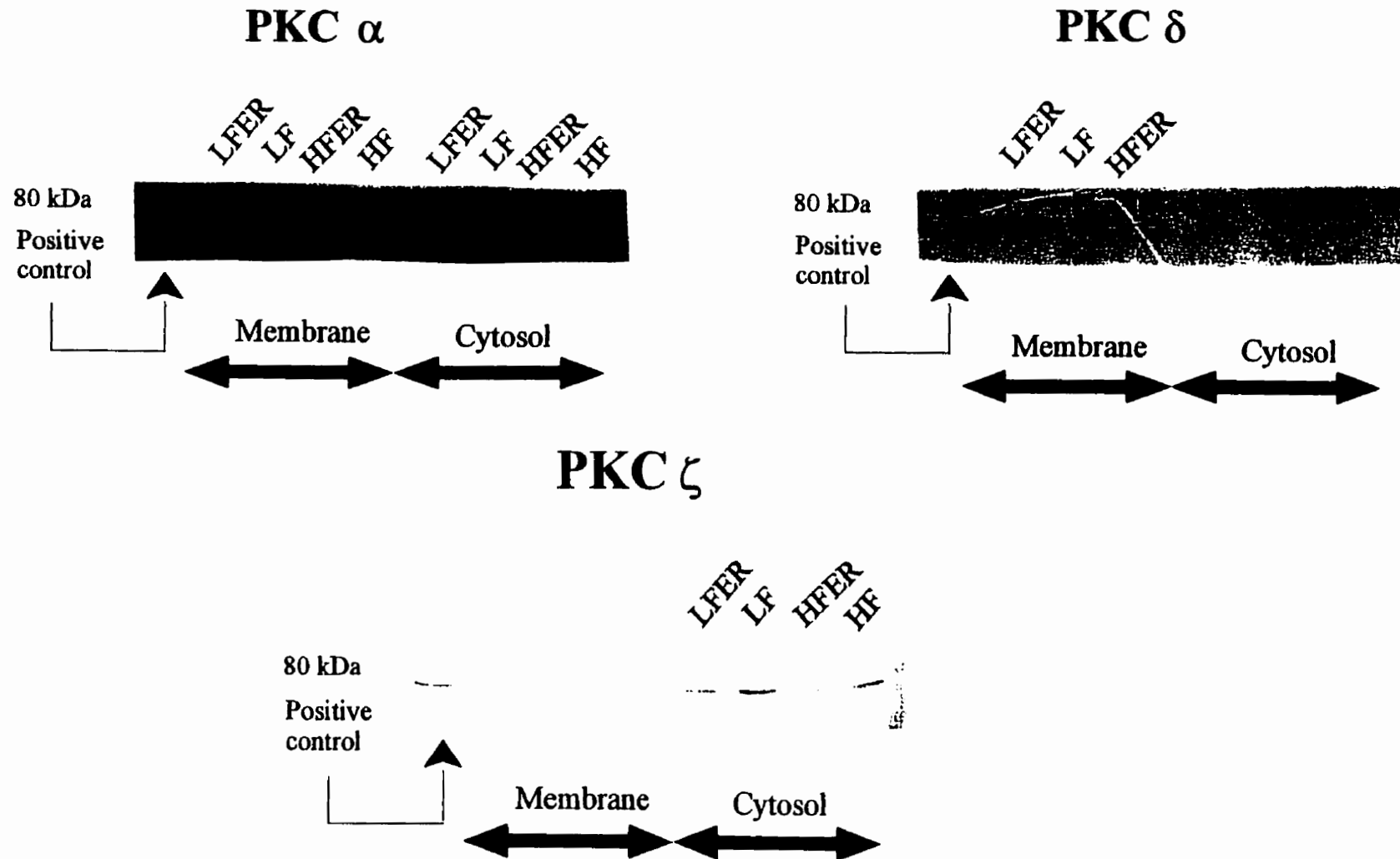
Table 9.2 Colonic mucosa and tumor EGFR-TK activity [1] in male F344 rats fed one of four diets, HF, HFER, LF, LFER and killed 28 weeks after carcinogen injection[2].

DIET		Stimulated EGFR-TK[3]	Non-stimulated EGFR-TK[4]
HF	mucosa	5.71 ● 0.44 [b]	0.21 ± 0.07 [b]
	tumor	3.15	5.29
HFER	mucosa	9.51 ● 1.63 [a]	0.57 ● 0.16 [b]
	tumor	4.38	1.19
LF	mucosa	4.85 ● 0.39 [b]	1.09 ± 0.33 [b]
	tumor	5.77	3.70
LFER	mucosa	6.00 ± 1.02 [b]	0.69 ± 0.11 [b]
	tumor	4.78	6.30
Average of all tumors		4.52 ± 0.54 [b]	4.12 ± 1.11 [a]

[1] Activity expressed as pmol Pi/min/100μg protein, values are means±SEM. Means in a column sharing a common superscript are not significantly different ($p \leq 0.05$).
[2] Diet treatments were initiated 16 weeks after injection.
[3] EGFR-TK activity as stimulated by previous incubation with TGF-α.
[4] EGFR-TK activity without previous incubation with TGF-α.

Table 9.3 Two-way ANOVA P values for EGFR-TK activity in male F344 rats fed one of four diets, HF, HFER, LF, LFER and killed 28 weeks after injection [1].

Variable	P values	
	Stimulated EGFR-TK[2]	Non Stimulated EGFR-TK[3]
FAT	0.0454	0.0200
ER	0.0253	0.9349
FAT x ER interaction	0.2054	0.0675

[1] Diet treatments were initiated 16 weeks after injection.
[2] EGFR-TK activity as stimulated by previous incubation with TGF-α.
[3] EGFR-TK activity without previous incubation with TGF-α.

average of tumors from all diet groups) was lower than mucosal activity in all diet groups. Non stimulated EGFR-TK activity was higher in the tumors compared to the mucosa from all groups . Consequently, fat was identified as the only significant variable affecting the non-stimulated activity (P = 0.0200, two-way ANOVA Table 9.3), with the higher activity in the low fat groups.

9.4   Discussion

The main findings of the present study are that ER and fat significantly affect EGFR-TK activity whereas neither variable significantly affected PKC activity. The PKC activity appears to be more variable than the EGFR-TK activity. High variability of PKC activity in normal and neoplastic human colonic mucosa has been previously reported and suggested to be one of the limiting factors in utilizing PKC activity as a marker for colorectal neoplasia (Levy et al., 1993;McGarrity and Peiffer, 1994). However, similar trends between the two assays are observed, mainly with the ER groups exhibiting higher activity than their respective counterparts.

PKC and EGFR-TK activity have not been previously assessed in colonic mucosa of rats at this late stage of carcinogenesis or in conjunction with each other. It has been reported that the EGFR-TK is highly stimulated by the binding of TGF-α to the EGF receptor (Malecka-Panas et al., 1996). Therefore it is of interest to determine the stimulated and non stimulated activity of the EGFR-TK. A previous investigation conducted by Kumar and colleagues (1990) examined general tyrosine kinase activity (not specific for EGFR-TK) in colonic mucosa five days after injection of AOM and in end stage tumors and demonstrated that tyrosine kinase activity was significantly lower in

HFER (30%) mucosa, and tumors compared to the HF counterparts. The results of the present study are paradoxical with the HFER (20%) exhibiting the highest activity. However, the differences in experimental protocol may in part explain the observed differences. Total TK was measured in the study by Kumar et al., (1990) where as the present study measured EGFR-TK only, which represents only one form of several tyrosine kinases within a cell. In addition, the activity measured in the present study was in colonic mucosa 28 week after injection, whereas the Kumar study (1990) measured activity 5 days after injection. The activity of the enzyme measured at the later stages of carcinogenesis may represent a more stable adapted response to changes in diet, where as the activity measured 5 days after injection may still be under the influence of the carcinogen and early responses to changes in diet.

Changes in enzyme activity may be the result of a plethora of possibilities such as; increased synthesis, increased co-factor requirement, decreased turnover rate, decreased or increased substrate availability. Therefore, the observed increases in EGFR-TK activity in the ER groups could be the result of several physiological events including; an increased growth state; increased EGFR-TK synthesis; decreased turnover rate; and increased receptors due to lack of ligand substrates.

Phosphorylation of proteins on tyrosine residues, through the activation of intrinsic tyrosine kinase of the EGFR, has been associated with mitogenic cellular responses (Ullrich and Schlessinger, 1990; Ushiro and Cohen, 1980). It has been previously demonstrated that the cellular turnover rates of the colonic mucosa is one of the highest in any tissue of the body (Schneeman, 1993). Therefore, a decrease in cellular

turnover could lead to a situation where the normal rate of sloughing off differentiated cells is altered. Such a phenomenon could lead to a build up of an abnormal cell mass. Therefore, increased activity of enzymes involved in cell growth and differentiation, such as PKC and EGFR-TK, could possibly be a positive response in attempts to reduce build up of old differentiated cells. This theory may, in part, explain the observed trend for increased activity of these enzymes in the HFER and LFER groups relative to their respective counterparts in the present study. Incidentally, Huang and Ives (1987) have previously demonstrated the occurrence of significant growth inhibition, as demonstrated by decreased mitogenesis, when PKC activity was maximal in vascular smooth cells. Moreover, it has been suggested that cells exhibiting PKC down-regulation may be more susceptible to transformation (Kusunoki et al., 1992). Conversely, as discussed in Chapter 5, the elevated activity observed in the assay could be an indication of prior inactivation *in vivo*.

Perturbation of the hormonal milieu in ER rats, compared to ad libitum, is another proposed explanation for the tumor inhibitory effects of ER. Several studies have demonstrated the ability of ER in rats to significantly alter the circulating level, binding capacity, and mRNA expression of several growth factors and hormones involved in regulating cellular metabolism (Breese et al., 1991; Clemmons and Underwood, 1992; Dauncey et al., 1994; Kalant et al., 1998; Oberkotter and Rasmussen, 1992; Ruggeri et al., 1989; Spindeler et al., 1990; Straues, 1994). In some cases, the ER rats demonstrated an increased binding capacity of a growth hormone to its receptor (Rugerri et al., 1989). It is well documented in human nutrition that cyclical ER may increase the efficiency of

conversion of food into stored energy (Brownell, 1987). In addition, the amount of

circulating TGF-α ligand may be reduced be ER. Considering the evidence in human and

rat studies, it may be postulated that the increased EGFR-TK activity in the present study

may be a result of an increased efficiency in binding capacity of TGF-α and consequent

activation of the EGFR-TK.

Observed increases in mucosal EGFR-TK activity may be an indication of several

physiological consequences and may not always be indicative of increased neoplastic

potential. Although EGFR-TK was elevated in both ER mucosa and tumors, increased

activity in the ER environment could be the result of a lack of ligand whereas increased

tumor activity could be an indication of an increased growth state. In addition, the

expression of enzyme activity in a certain amount of protein could be misleading if the

interest is in activity as the cellular level. Tumor cells contain more protein/cell than

normal cells (R.P. Bird, personal communication) which would underestimate the total

activity per total protein in tumor cells. The observation of higher activity in the non-

stimulated environment for some of the tumors could be the result of this particular

experimental protocol. Fractions were incubated with TGF-α for a designated period of

time. If the activity was assessed in a timed response manner (i.e. sequential analysis of

activity from 1-15 min.) it may have been noted that tumor EGFR-TK activity was higher

during the early stages of incubation.

It is also important to note that the nature of the experimental protocol of the

present study (detailed in Chapter 8) is very different from most diet intervention studies

in carcinogen treated colonic mucosa. The changes in enzyme activity reflect the ability of

diet to modulate activity several weeks after carcinogen treatment. Changes in dietary fat and energy content were implemented roughly half way through colon carcinogenesis in rats at least 20-22 weeks old, whereas most studies implement changes in diet before or shortly after carcinogen injection among rats at a considerably younger age.

Similar to the previous studies (Chapters 4 and 5) the expression of PKC isoforms did not always corroborate the trends observed in PKC activity. As mentioned previously, the measurement of two isoforms out of a family of twelve, may not accurately represent the expression of all isoforms at the protein level.

As mentioned previously, the isoform expression in tumors is merely the first attempt to process tumors with this method of separation and the results must be taken with caution. However, the absence of PKC δ in the cytosol and the absence of PKC ζ in the membrane may suggest divergence in the expression of PKC isoforms in colonic tumors. PKC ζ has recently been demonstrated to be the only isoform that is not translocated from the cytosol to the membrane by a particular phorbol ester in rat colonocytes (Bissonnette et al., 1995). This data sheds light on the observation of the present study. The lack of PKC ζ in the membrane fraction of the present study may indicate that this isoform is not activated due to its lack of translocation from the cytosol. Interestingly, this isozyme has also been demonstrated to be mainly cytosolic in a number of cell lines from tissues other than colon (Hug and Sarre, 1993). In addition, Craven and DeRubertis (1994b) have observed a variable and decreased immunoreactive response to PKC δ in human adenocarcinomas. It has been suggested that depending on the cell type, PKC δ appears to be differentially distributed within the cells (Hug and Sarre, 1993).

Therefore, the absence of PKC δ in the cytosolic fraction of tumors is not surprising. Moreover, PKC δ has been purported to inhibit proliferation (Goodnight et al., 1995) while PKC ζ has been suggested to be a critical isoform in mitogenic signal transduction (Berra et al., 1993). Such data provides the basis for additional studies in which tumor PKC isoform expression as detected by immunoblotting may be further analysed in several tumors.

The carcinogen treated colonic mucosa is a combination of millions of normal crypts and perhaps thousands of ACF. The method of mucosal preparation in the present study results in a mixture of these two phenotypes. Therefore a limitation of the present study stems from the fact that the resulting activities and isoform expressions are not an indication of one particular population but rather an indication of the colonic mucosal activity and isoform expression as a whole. Further work involving *in situ* protocols and microdissection of specific crypt populations may circumvent this limitation.

In summary the activity enzymes involved in cell growth and differentiation measured in mucosa at the later stages of colon carcinogenesis were altered by changes in diet that were implemented half way through the colon carcinogenic process. ER appears to enhance the activity of these enzymes which could be a reflection of prior inactivation, or as an adaptive response to enhanced turn over rate of colonic cells. The significance of increased or decreased PKC and/or EGFR-TK activity in relation to end tumor incidence may be further elucidated by sequential analysis of enzyme activity at several time points during the varying stages of colon carcinogenesis. Due to the high variability of PKC activity , its viability as marker for colorectal neoplasia may be limited. Future

studies exploring the divergence of PKC isoform expression in rat colonic tumors may

provide further knowledge towards the significance of this family of enzymes in colon

tumorigenesis.

10. DETECTION AND SEMIQUANTITATIVE ANALYSES OF PKC ISOFORM , EGFR AND SRC mRNA EXPRESSION BY RT-PCR IN NORMAL COLONIC MUCOSA, ACF , MICROADENOMAS AND TUMORS

10.1 Introduction

The proposed genetic model for colo-rectal tumor development (Vogelstein et al., 1988) has created a vast interest in further identification of modified gene expression pattern in normal versus preneoplastic (Stopera et al., 1992; Pretlow et al., 1992) and neoplastic colonic tissues (Levy et al.1993; Davidson et al., 1994). With the advent of improved techniques in molecular biology (Ausubel et al., 1995) rapid identification and quantification of altered gene expression has become a key facet in the emerging research.

Early detection of altered gene expression may play a key role in identifying and treating the disease before tumor development. Therefore, several studies have explored genetic aberrations in preneoplastic ACF. ACF have been demonstrated to exhibit ; mutations in the p53 gene (Stopera and Bird, 1993); elevated expression of c-*fos* (Stopera et al, 1992); mutated K-*RAS* expression (Pretlow et al., 1995; Stopera et al., 1992; Shivapurkar et al., 1994; Singh et al., 1994; Stopera et al., 1992; Tachino et al., 1995;Vivonia et al., 1994; Zaidi et al., 1995); and DNA adduct formation (Qin et al., 1994). Such studies lend support to the contention that ACF are preneoplastic lesions which exhibit genetic alterations common to end stage tumors (Fearon and Vogelstein, 1990).

The preceding chapters have eluded to the potential alterations in PKC isoform

expression and PKC, tyrosine kinase and EGFR-TK activity in carcinogen treated colonic mucosa harbouring a mixture of normal and aberrant crypts. In order to distinguish the potential differences between the two populations a method must be utilized in which ACF and normal crypts are analysed independently.

The present study was designed to use a novel method by which alcohol fixed ACF are micro dissected from normal surrounding crypts and analysed for specific mRNAs by RT-PCR, a technique which has not been previously attempted. Specific objectives of the investigation include; 1.) successful microdissection and RT-PCR of alcohol fixed ACF , normal crypts, microadenomas and tumors; 2.) semiquantitative measurement of mRNA expression of PKC isoforms in each tissue type; 3.) semiquantitative measurement of mRNA expression of EGFR and SRC in each tissue type.

10.2   Materials and Methods

Preparation of Tissues for RNA extraction

To determine the optimal method of tissue fixation and preservation for successful RT-PCR, trial colonic mucosa was treated in several different ways. Trials were completed on fresh tissue (non fixed non frozen), frozen non-fixed, and frozen formalin fixed tissue. Formalin fixed tissue did not yield any mRNA. Fixation and freezing in alcohol did not alter the yield of mRNA as compared to fresh tissue(data not shown). Therefore, all tissues were fixed in 70% ethanol and frozen at -80°C.

All procedures were carried out under RNASE-free conditions using sterile gloves, autoclaved solutions, filter papers and diethylpyrocarbonate (DEPC, appendix C) treated dissecting instruments. Approximately 5 animals from each diet group were used at the final termination point (28 weeks) from the study detailed in Chapter 8. Animals were terminated by CO_2 asphyxiation. Colons were excised, slit from cecum to anus, and flushed with RNASE-free ice cold phosphate-buffered saline (PBS). Colons were then placed flat on filter paper and fixed in 70% ethanol and immediately frozen at -80°C. Fixation of the colon was conducted on an RNASE-free cold plate maintained at 4°C. Colons were removed from the -80°C freezer and placed on the lid of a sterile, clear petri dish filled with ice to insure a cold surface for micro-dissection. Tumors and microadeomas were quickly excised from the mucosa. Classifications used to distinguish tumors from microadenomas were as described in chapter 8. The petri dish was then immediately placed under a micro-dissecting microscope at 10-20X magnification. Colonic crypts not displaying any abberant features were dislodged from the mucosa from areas along the entire length of the colon. ACF consisting of greater than 4 crypt/focus were plucked quickly from the normal surrounding mucosa using DEPC treated and autoclaved micro-dissecting forceps (FWR #55 Dumont Bio Inox Forceps, 0.05* 0.02mm tip).

RNA Extraction Procedures

All reagents and enzymes were obtained from Gibco BRL (Burlington, Ontario, Canada) unless specified otherwise. Extraction of RNA was based on the method developed by Chomczynski and Sacchi (1987). Tumors and microadenomas were placed

in 200-400 µl denaturation solution (4M guanidine thiocyanate, 25mM sodium citrate,pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Approximately 500 normal crypts and 500 ACF were placed into 100- 200 µl denaturation solution. The samples were power homogenized in 1.5 ml conical microcentrifuge tube with a tissue grinder pestle (Kontes #749515-000) for 30 seconds. 1/10 volume 2M sodium acetate pH 5.2 and 1 volume water saturated ultra pure phenol with 0.1% hydroxy quinoline (W/W) were added, vortexed, then incubated at 60°C for 10 minutes. 1/5 volume of chloroform-isoamyl alcohol (49:1, v/v) was added, vortexed then incubated on ice for 30 minutes. During this incubation, the suspension separates into the top aqueous phase and the bottom organic phase. The suspension was centrifuged 30 minutes at 16,000x g at 4°C. The aqueous phase was transferred to a new tube with an equal volume of chloroform-isoamyl alcohol (29:1), vortexed then centrifuged for 10 minutes at 16,000 x g at 4°C. The aqueous phase was transferred to a fresh tube and mixed with 2 volumes absolute ethanol and placed at -80°C for a minimum of 30 minutes to precipitate the RNA. The RNA was recovered by centrifuging at 16,000 for 30 minutes at 4°C. The RNA pellets were washed by adding 400µl 80% ethanol, centrifuging 10 minutes at 16,000 g at 4°C. The ethanol was removed and the pellets were air-dried at room temperature for 15 minutes. The pellets were resuspended in 33µl autoclaved ultra pure deionized water. 37U DNAse 1 and 38.9U RNA guard (Pharmacia, Quebec, Canada) were added to a solution consisting of 40mM Tris pH 7.5 and 6mM $MgCl_2$ and incubated at 37°C for 60 minutes in a volume of 50µl. The DNase was inactivated by 100mM EDTA, then phenol extracted and ethanol precipitated. The RNA pellet was resuspended in 15-30 µl autoclaved, ultra pure

deionized water. The concentration was determined by the absorbance at 260 nm.

cDNA synthesis by Reverse Transcription

Reverse transcription was performed on 1 μg total RNA in a 20 μl reaction

volume according to the methods detailed by Gibco BRL. Initially, 1 μg of RNA, 8 μl of

H_2O and 1 μl of oligo dt (500 μg/ml) were combined and heated at 65°C for 10 minutes,

followed by 5 minutes on ice. Following the incubation on ice was the addition of 1 μl

(39U) of RNA guard (Pharmacia), 2 μl of 100 mM DTT, 2 μl of 5mM dNTP and 4 μl of

5x first strand buffer (appendix C). The mixture was vortexed and centrifuged briefly.

Reverse transcriptase was performed by the addition of 1μl (200U/μl) of M-MLV

(Moloney Murine Leukaemia Virus) at 42°C for 2 hours.

Polymerase Chain Reaction

The sources of the primer sequences and their characteristics are detailed in Table

10.1. Once the sequences were obtained, the primers were synthesized by Gibco Life

Technologies. PCR was carried out using a PTC-100 Thermocycler (MJ Research Inc.)..

In attempt to semiquantify PCR products, the primer dropping method (Wong et

al., 1994) was employed in order to co-amplify the product of interest (PKCs,

EGFR,SRC) and the housekeeping gene β-actin. The expression of the β-actin gene is

reportedly equal from one cell to another (Wong et al., 1994). Therefore, the ratio of

expression of the amplified PCR product of interest (PKC isoform, EGFR, SRC) to that of

β-actin was used to determine differences from one group to another.

Following cDNA synthesis, 1.6 μl of the cDNA reaction mix was amplified in a 50

μl PCR reaction in two 25 μl steps. The initial 25 μl reaction mix contained 1.6 μl of

cDNA, 16.96 µl H$_2$O, 2.50 µl 10x PCR buffer (appendix C), 2.50 µl 2 mM dNTP, 0.75 µl

50 mM MgCl$_2$ 0.50 µl 25 pmol PKC (α,β1,δ,ε,ζ), EGFR or SRC 3' and 5' primers and

0.1875 (5U/µl) Taq DNA polymerase. The reaction was mixed gently and overlayed with

mineral oil (Sigma) and preheated for 2 minutes at 94°C then for 2-15 (cycle number

depending on the type of primer) cycles at 94°C 1 minute, 52°C 2 minutes, 72°C 3

minutes. After these initial cycles, a maintenance stage at 80°C for 10 at the end of the

last cycle to allowed for addition of the second stage reaction cocktail. This second

reaction cocktail was identical to the previously described cocktail with the addition of 50

pmol of 3' and 5' β-Actin primers. After the addition of the second stage cocktail the

mixtures were then subjected to another 28 cycles of PCR amplification as described

above and maintained at 4°C until storage at -20°C. As a control to check for any

endogenous sources of DNA contamination, PCR was performed on the reaction cocktail

identical to all other samples without the addition of sample cDNA.

Visualization and Quantification of PCR products

The PCR products were analysed on a 2% agarose gel in TBE buffer (see

appendix C) using the Gibco BRL Horizon 11-14 gel electrophoresis apparatus at

100volts for 1 1/2 hours. The resulting gels were photographed under uv illumination

with Polaroid film. The resulting photographs were scanned using a Reliasys Image

Scanner and Photoshop version 2.5 software for Macintosh. The area of the product

bands (mm^2) detected on the film was then calculated using Image version 1.49 software.

The area of the band corresponding to the particular primer was expressed as a ratio

relative to the area of the band corresponding to β-actin. β-actin is considered to be a

Table 10.1   Primers for RT-PCR

		PRIMER SEQUENCE	Product size	Number of Cycles	TM	%GC content
PKC α[1]	Forward	5'-TGAACCCTCAGTGGAATGAGT-3'	325	33	65	47
	Reverse	5'-GGCTGCTTCCTGTCTTCTGAA-3'			67	52
PKC β1[2]	Forward	5'-CCCGGAGTGGAACGAAACCTTC-3'	300	43	70	59
	Reverse	5'-TCCGGTCCCTGTTGCCATTG-3'			68	60
PKC δ[1]	Forward	5'-CACCATCTTCCAGAAAGAACG-3'	352	31	65	47
	Reverse	5'-CTTGCCATAGGTCCCGTTGTTG-3'			68	54
PKC ε[1]	Forward	5'-CGAGGACGACTTGTTTGAATCC-3	389	30	67	50
	Reverse	5'-CAGTTTCTCAGGGCATCAGGTC-3'			68	54
PKC ζ[1]	Forward	5'-CGATGGGGTGGATGGGATCAAAA-3'	686	31	68	52
	Reverse	5'-GTATTCATGTCAGGGTTGTCTG-3'				
EGFR[3]	Forward	5'-TAGAAATGGGGAGCTGCCGTGTC-3'	220	33	68	54
	Reverse	5'-AGGGTTGCTCACCGCATTG-3'			66	57
SRC[4]	Forward	5'-GCTGCAGGTTTGAAGCTGGGT-3'	762	46	68	57
	Reverse	5'-CGGTCCTCTGCCACGTAATTG-3'			68	57
β-ACTIN[5]	Forward	5'-CTCCTTAATGTCACGCACGATTTC-3'	541	28	67	45
	Reverse	5'-GTGGGGCGCCCCAGGCACCA-3'			75	80

[1] Davidson et al., (1994), [2] Housey et al., (1988) [3] Carroll et al., (1996) [4] Avigan, (1994)
[5] Huang et al., (1995).

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"housekeeping gene" which is present in equal amounts in all cells (Wong et al, 1994) and has recently been used in conjunction with PKC δ primers in rat colonic neoplasms in attempts to quantify PCR products (Yoshimi et al., 1994).

Statistical analysis

Statistical analysis of PCR products were conducted by ANOVA with Duncan's multiple range test. A P value ≤ 0.05 was considered significant.

10.3 Results

The most tedious aspect of the isolation of genomic RNA is to avoid degradation by endogenous RNAses. A great deal of care was taken to use RNAse free conditions. As expected isolation of intact genomic RNA from alcohol fixed tissue was successful since alcohol does not affect its structural integrity.

A sample consisting of 100-150 ACF consisting of greater than 4 crypts/focus yielded an average of 9.46 μg of RNA for RT-PCR. Normal colonic crypts sample of a population of approximately 500 crypts yielded a similar concentration of RNA. Analyses could be carried out successfully on 4 out of 5 ACF samples (80%). Analyses of microadenoma , tumor and normal mucosa RT-PCR was successful for 100% of the samples processed. The approximated error between repeated cDNA and PCR reactions was approximately 5% and 14%respectively. For each primer, PCR was conducted on at least 3 ACF populations (500 crypts each), 3 normal populations (500 crypts each), 4 microadenomas and 4 tumors. Figure 10.1 details an example of the resulting primer products for EGFR and SRC relative to β-actin for populations of normal crypts, ACF, microadenomas and tumors.

Expression of PKC isoforms

Analysis of the resulting PCR products for the PKC isoforms are detailed in Figure

10.1. The results are expressed as a ratio of particular isoform expression to β-actin

expression. PKC isoform expression was not significantly different among the colonic

tissues. Although not statistically significant several trends may be observed. The

microadenomas appeared to exhibit a trend for highest expression for four out of the five

isoforms. Relative to normal mucosa, the tumor and ACF populations expressed similar,

if not slightly lower, PKC isoform expression.

Expression of EGFR

Figure 10.2 details EGFR expression analysis for the various colonic tissues.

Similar to the PKC isoform expression, statistical significance was not observed.

However, the order of EGFR expression from highest to lowest was tumors >

microadenomas> normal >ACF.

Expression of SRC

SRC expression analysis is detailed in Figure 10.3. ACF exhibited a significantly

higher level of SRC expression that the rest of the tissues. The absolute value calculated

for average ACF was 9.03±2.97 which was more than four times the next highest tissue,

that being the tumors. Figure 10.4 is a representative photograph of a 2% agarose gel in

which SRC and β-actin primer cDNA products were separated. Although not statistically

significant, the microadenomas and normal crypts exhibited slightly lower levels of

expression than the tumors. PCR on the reaction mix cocktail and various primers did not

reveal any DNA contamination (data not shown).

Figure 10.1 RT-PCR of PKC isoforms DNA fragments in normal crypts, ACF,

microadenomas and tumors. Values are expressed as a ratio of the areas of the

visualised product bands for the PKC:β-actin primers. Values are means $\pm$SEM(bars).
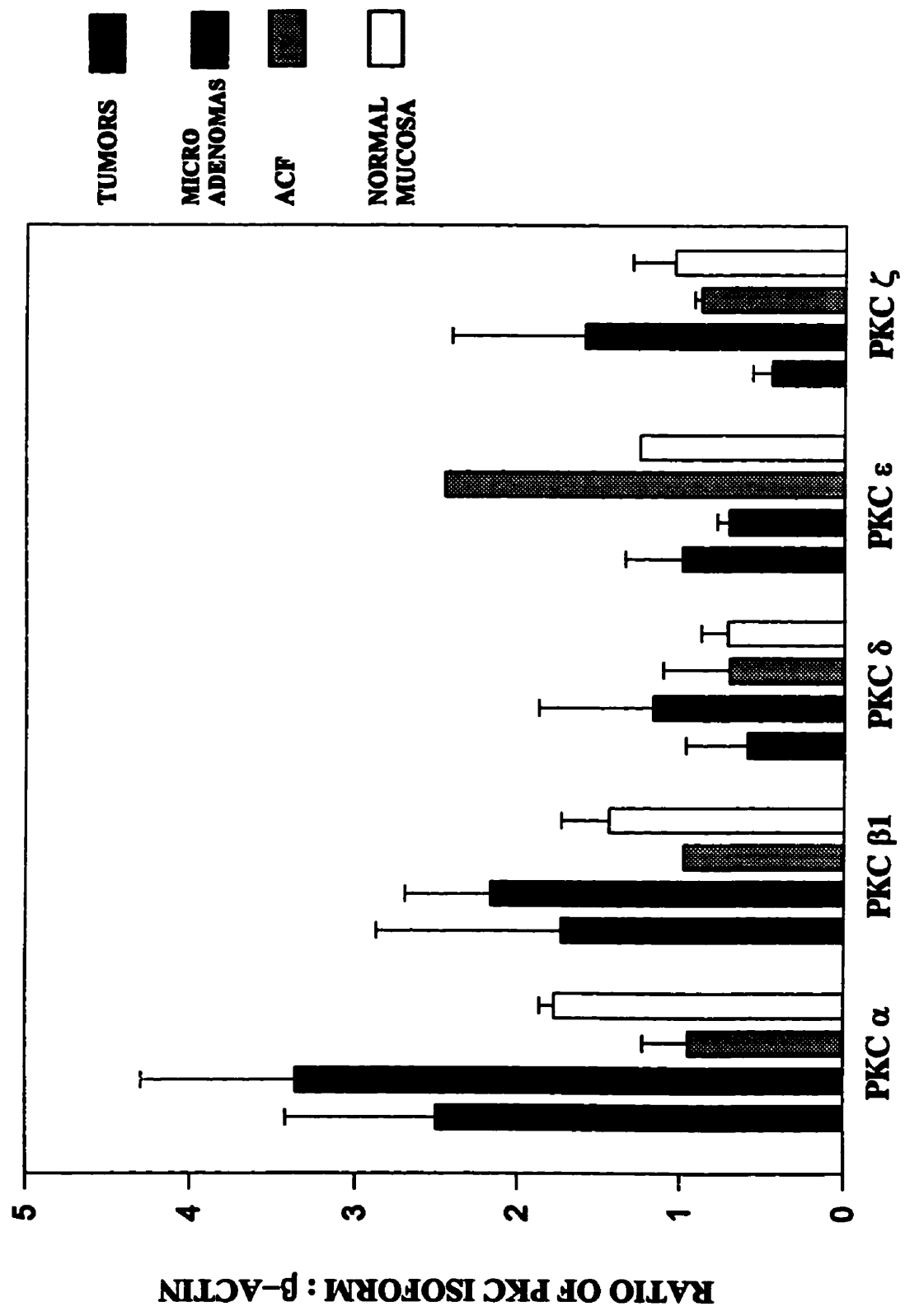
Figure 10.2   RT-PCR of EGFR DNA fragments in normal crypts, ACF,

microadenomas and tumors. Values are expressed as a ratio of the areas of the

visualised product bands for the EGFR:β-actin primers. Values are means

$\pm$SEM(bars).

RATIO OF EGFR:β-ACTIN

TUMORS

MICRO ADENOMAS

ACF

NORMAL MUCOSA
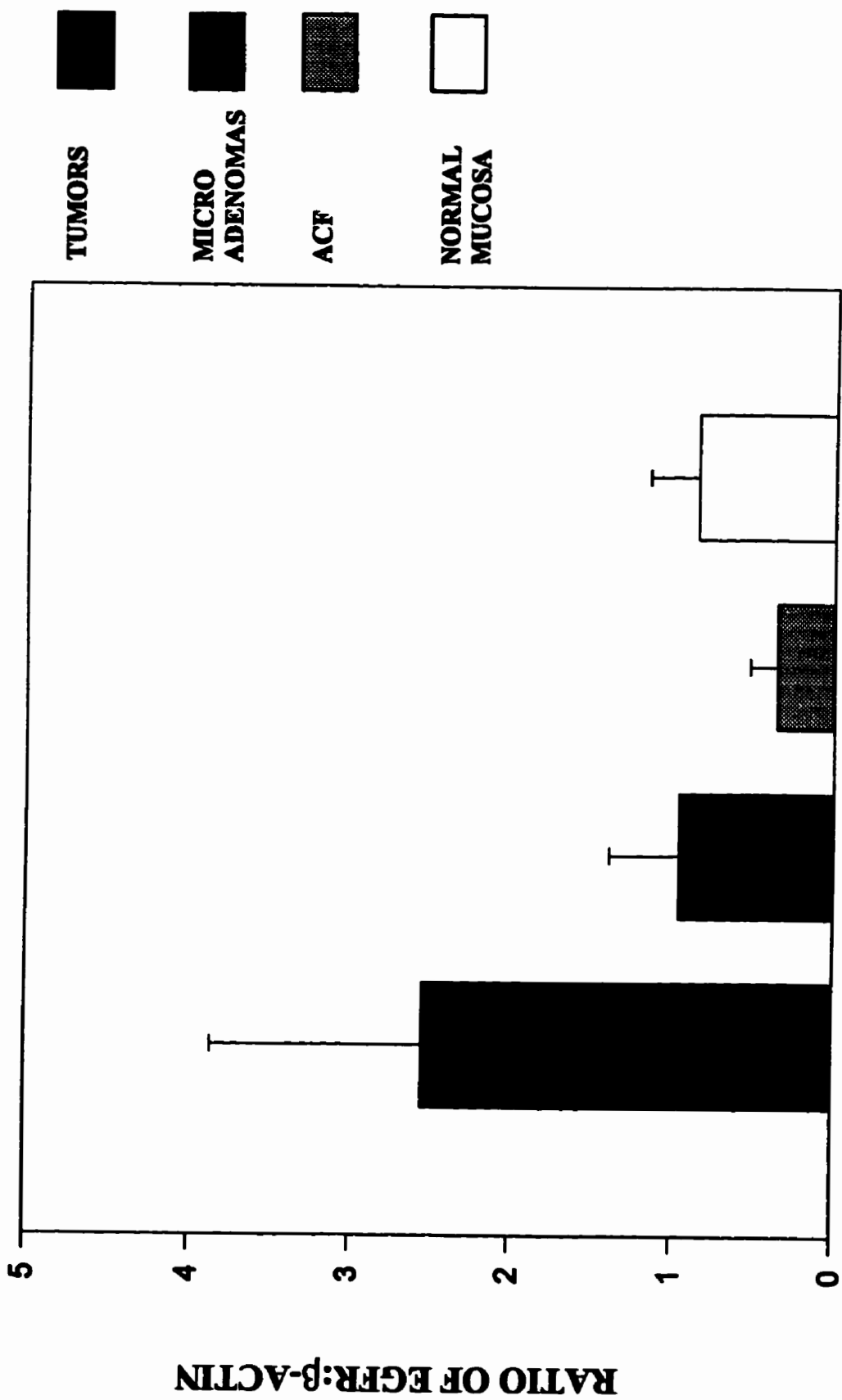
Figure 10.3 RT-PCR of SRC DNA fragments in normal crypts, ACF, microadenomas and tumors. Values are expressed as a ratio of the areas of the visualised product bands for the SRC:β-actin primers. Values are means $\pm$SEM(bars). •Denotes significant difference P$\leq$0.05, ANOVA with Duncan's multiple range test.

RATIO OF SRC:β-ACTIN

TUMORS

MICRO ADENOMAS

ACF

NORMAL MUCOSA
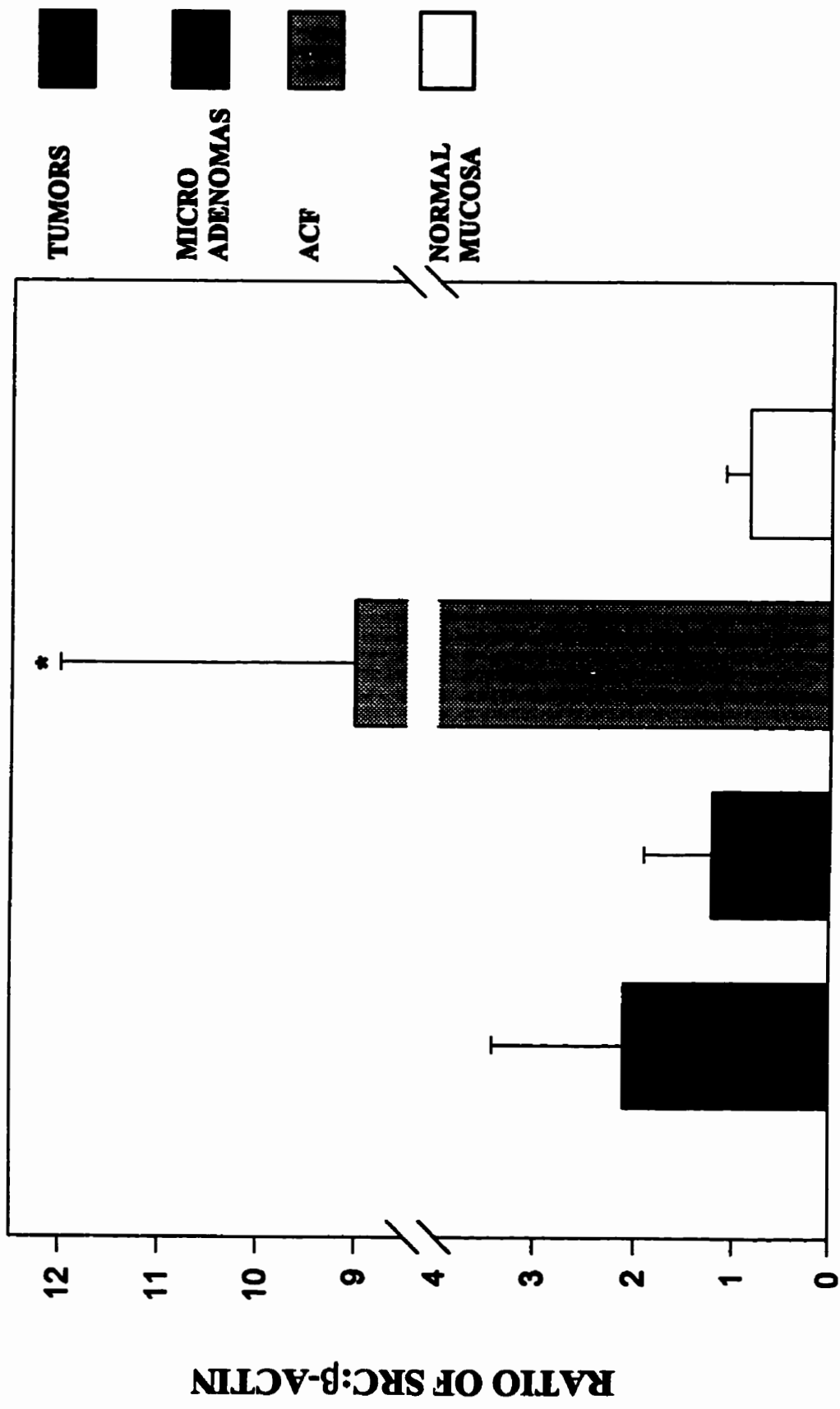
Figure 10.4 2% Agarose gel analysis of SRC DNA fragments by RT-PCR.

10.4 Discussion

This approach provided the unique opportunity to explore gene expression in

ACF. To the author's knowledge, this was the first attempt to measure gene expression

in micro dissected preneoplastic colonic ACF from alcohol fixed tissue. Successful micro

dissection of ACF under completely RNAse free conditions was one of the major goals of

the present study.

A limitation of this approach is that the analyses was carried out on a pooled

sample of ACF. However this limitation was minimized by isolating the population of

ACF of similar growth characteristic, that being greater than 4 crypts per focus. This is an

important point to consider if different preneoplastic stages are to be investigated and that

experimental evidence suggests that crypt multiplicity of ACF represent its preneoplastic

state (Magnuson et al., 1993, Zhang et al., 1992).

The purported housekeeping gene β-actin was utilized in attempts to

semiquantify PCR products. Although this method has been used previously in rat colon

neoplasms (Yoshimi et al., 1994), it has been demonstrated that mRNA levels of β-actin

and another commonly used housekeeping gene, known as glyceraldehyde-3-phosphate-

dehydrogenase (GADPH), may have significant inter-tissue and inter-individual variation

in rat liver, spleen and brain (Slagboom et al., 1990). Incidentally, the inter-individual

variations of these genes has not been explored in colon. However, the use of these genes

as internal standards for quantitative PCR continues. Several other methodologies for

quantitative PCR are emerging (Siegling et al, 1994).

The small sample sizes of each tissue type may have prevented the observation of

significant differences in mRNA expression for the genes of interest. However, the observed trends appear to reflect those presented in previous investigations. PKC isoform expression does appear to be consistently elevated in tumors, which has been demonstrated previously (Davidson et al., 1994; Levy et al., 1993). The notably trend for the microadenomas to express higher levels of almost all isoforms in relation to all other tissue is quite interesting. As previously discussed, the down regulation of PKC activity and perhaps PKC mRNA, in tumors may be a result of prolonged activation (Craven and DeRubertis, 1992a). There has been no attempt to explore the profile of PKCs in microadenomas. Perhaps the elevated levels of PKC mRNAs in the present study reflect the stage at which PKC expression is maximal and precedes the down regulation observed in tumors. Elevated PKC expression may be a requirement for the formation of microadenomas which may subside upon obtaining a certain level of growth autonomy allowing them to proceed to the tumor stage. Therefore, elevated PKC expression may be optimal at transitional stages of development.

With the exception of PKC ϵ, ACF exhibited the lowest level of PKC isoform expression as compared to other tissues, including normal mucosa. This was the first attempt to explore PKC isoform expression in ACF, therefore the results cannot be discussed relative to previous findings. It is interesting to note that both ACF and tumors appeared to have down regulated expression relative to microadenomas and normal mucosa. Such observations support the hypothesis that PKC expression may be maximal in certain stages throughout the transition from normal to preneoplastic to neoplastic stages of colon carcinogenesis.

Significantly elevated SRC expression of ACF was not surprising considering the reportedly elevated levels of SRC activity in premalignant epithelia of ulcerative colitis (Cartwright et al., 1994) which harbours thousands of dysplastic colonic crypts. However, mRNA levels of SRC have not been previously investigated in preneoplastic colonic mucosa. Such observations suggest that elevated SRC mRNA expression and activity may be an important event during the early stages of colon carcinogenesis. Consequently, elevated SRC activity has been reported in malignant tumors (Cartwright et al., 1993;1994) suggesting that unlike PKC, elevated SRC activity may not undergo down regulation throughout the varying stages of carcinogenesis.

EGFR expression was not significantly different among the tissues suggesting that the amount of this membrane receptor involved in signal transduction may not change during the varying stages from normal to neoplastic colonic development. However, this receptor harbours intrinsic tyrosine kinase activity which may be itself be affected during colon carcinogenesis (Chapter 9).

The present study successfully used a novel method for RT-PCR on micro-dissected ACF. It was demonstrated that PKC isoform expression is variable and potentially down regulated in tumors and in ACF. SRC expression is elevated in preneoplastic ACF and EGFR expression may not be affected throughout varying stages of colon carcinogenesis. This study provides the foundation for future studies in which specific populations of ACF and tissues extracted from varying time points which precede tumor development may be analysed for various gene products.

11. GENERAL DISCUSSION AND CONCLUSIONS

Specific results of each study in this dissertation have been discussed in detail in their respective chapters. The focus of the present chapter will be to discuss the results in context of their contributions towards further understanding the multi-step process of colon carcinogenesis.

Mechanisms by which tumor modulators affect the disease outcome are often unable to identify. Investigations which explore the effects of tumor modulators in non-carcinogen treated colonic mucosa may indicate initial, or "base line", cellular changes which may or may not be dependent on carcinogen treatment. Chapters 4 and 5 served as such studies. In both studies, ER did not consistently alter PKC activity or isoform expression. Age appeared to be the main variable affecting PKC and tyrosine kinase enzyme activity. The high level of variability observed in PKC activity in both studies has also been demonstrated in human colonic adenomas (Craven and DeRubertis , 1992). These findings generate several implications. Firstly, the inability of these enzymes to be altered by changes in diet composition, which have been previously identified to be modulators of colon carcinogenesis, suggests that modulation of their activity in colonic mucosa may not be an important event prior to initiation of the disease. Secondly, the high variability and lack of consistent diet effect on PKC activity questions the validity of PKC as a risk marker for enhanced malignant potential in stages prior to initiation of the disease.

The most important findings of this dissertation are those detailed in chapters 6-

8 which collectively demonstrated that changes in dietary fat and energy modulated specific phenotypic populations of ACF, microadenomas and tumors and that the time of dietary intervention had a significant impact on the ability of the disease to be modified.

Colonic lesions were allowed to develop in low and high fat environments before being intervened with ER. It is important to expose the developing lesions to a high fat environment to determine if ER is an effective modulator of the growth of preneoplastic lesions with established phenotypes. This dissertation has clearly demonstrated and confirmed the purported existence of biological heterogeneity in both preneoplastic and neoplastic colonic lesions. These studies have addressed the questions which formulated the hypotheses of the dissertation.

At what stage of the multi-step process of colon carcinogenesis do changes in dietary fat and ER exert measurable responses? Studies 6-8 imply that the ability of the disease to be modulated is greatly affected by the stage at which the diets are changed with respect to fat and energy. Changes in diet composition soon after carcinogen injection, during the early stages of carcinogenesis, identified fat to be a more rapid modulator of ACF development than ER (Chapter 6). When diet intervention was introduced at the intermediate stages, following exposure of the early stages to low fat diets, fat and ER significantly affected ACF and microadenoma development (Chapter 7). As in Chapter 6, fat exerted measurable responses more rapidly than ER at these intermediate time points in Chapter 7. Chapter 8 demonstrated

the inability of changes in fat and ER to significantly alter total end tumor incidence when diet intervention was introduced during the later stages of carcinogenesis with prior exposure of the early stages to high fat diets.

Culmination of this data implies that intervention of the disease by changes in dietary fat and energy is more effective if introduced at the early stages of colon carcinogenesis. Feeding a high fat diet during the early stages (Chapter 8) appeared to impart a level of growth autonomy on the developing preneoplastic lesions enabling them with the potential to develop into neoplastic lesions that could not be affected by changes in diet. However, when the early stages were exposed to a low fat diet (Chapter 7) the level of growth autonomy attained was not sufficient to overcome the changes in fat and energy introduced at the intermediate stages. A recent study conducted in our laboratory demonstrated similar findings with respect to changes in dietary fat content (Bird et al., 1996).

The findings of the present dissertation regarding these issues provide substantial evidence supporting the contention that preventative strategies for altering the disease process are more effective when introduced at the early stages of disease promotion and progression.

Are specific phenotypic populations of ACF, microadenomas, and tumors selectively affected by changes in dietary fat and energy? The concept of biological heterogeneity among microadenomas and tumors is readily accepted. However, the existence of biological heterogeneity among ACF is a concept that has been addressed in relatively few studies (Magnuson and Bird., 1993; Bird et al., 1996). Changes in

the total number of ACF may not provide sufficient insight towards the potential effects of a particular modulator of the disease. Crypt multiplicity has been indicated to be the parameter associated with the ability to predict end tumor incidence (Magnuson et al., 1993; Zhang et al., 1992). When ACF are grouped into populations according to their crypt multiplicity, the effects of a particular modulator may be limited to a specific population of ACF. Chapter 6 revealed that the lesions most affected by changes in ER were those containing 4-6 crypts per focus (medium ACF). Chapter 7 demonstrated that ER within HF and LF diets affected different populations of ACF. The HFER diet inhibited the development of small ACF (1-3 crypts/focus) where as the LFER diet inhibited the development of medium ACF (4-6 crypts /focus). Chapter 8 also demonstrated the ability of fat to reduce the number of small and medium ACF without affecting the number of large ACF. Consequently, ER was not identified as a significant variable affecting any population of ACF within chapter 8.

These studies clearly demonstrate the heterogeneity among ACF in response to growth modulation by diet. In addition, the effects vary from one study to another, suggesting that these effects are also dependent on experimental protocol.

With respect to microadenoma and tumor development, Chapter 7 demonstrated the ability of ER within a high fat diet to significantly inhibit the conversion of microadenomas to tumors. Chapter 8 demonstrated that ability of a LF diet to enhance the development of microadenomas, yet did not increase the development of tumors.

Dietary fat and ER individually, modulate the disease in a distinct manner. In both situations, when the disease process was allowed to develop in low and high fat

environments, fat and ER exerted their effects independently without any significant interactions.

This dissertation demonstrates that changes in diet affect varying populations of microadenomas and their development into tumors, substantiating the presence of biological heterogeneity. Similar to the modulation of ACF, the effects of diet on microadenomas and tumors are dependent on the experimental protocol.

Biological heterogeneity among ACF has been demonstrated previously in relation to dysplastic changes (McLellan et al., 1991a). However, this dissertation substantiates the ability of particular changes in diet to endow a particular level of autonomy on certain populations of ACF creating biological heterogeneity with respect to growth autonomy. Figure 11.1 (Bird, 1995) depicts a scheme detailing the multi step process of colon carcinogenesis. This scheme proposes that only a select few ACF with a certain level of autonomy will progress onto microadenomas and adenomas. This scheme also proposes that the growth of precursor lesions can be modulated at different stages.

Growth rates of lesions may also display heterogeneity. For example, not all ACF consisting of 1 crypt will convert into an ACF with 2 crypts. Certain lesions could require a longer duration to progress to a lesion of higher multiplicity. This concept also applies to microadenomas and tumors. Not all microadenomas convert into tumors, a rapid growth rate could equip certain populations of microadenomas with the ability to progress into a tumor. Once these lesions reach a certain size or phenotype, they may regress or partially or fully differentiate (remodel).

Figure 11.1 Proposed scheme depicting the multistage process of colon carcinogenesis

by Bird (1995). The scheme represent a model by which the selective growth

advantage of only a few precursor lesions which can be modulated at different stages of

colon carcinogenesis.

ABERRANT CRYPT FOCI

ABERRANT CRYPT FOCI WITH
GROWTH AND MORPHOLOGICAL
HETEROGENEITY

MICROADENOMA

ADENOMA
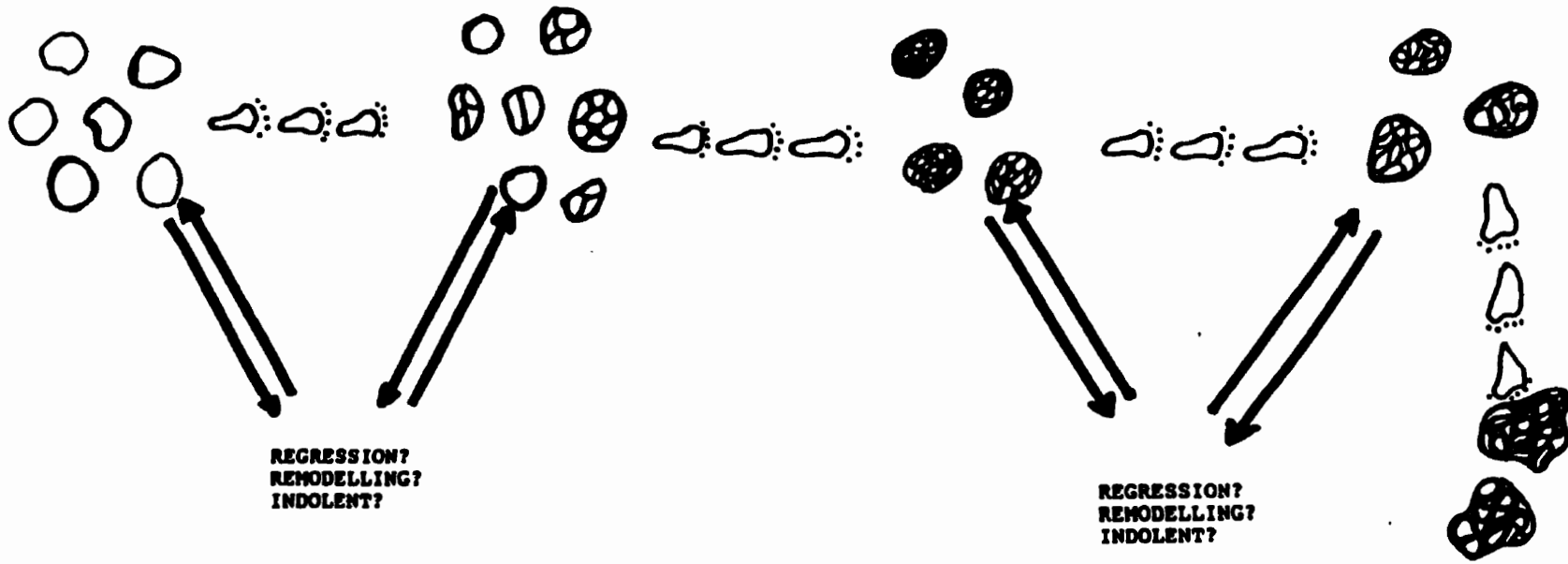
REGRESSION?
REMODELLING?
INDOLENT?

REGRESSION?
REMODELLING?
INDOLENT?

ADENOCARCINOMA

In concert with the concepts proposed in this scheme and the findings detailed in this dissertation dietary fat and energy are significant modulators of colon carcinogenesis. In addition, they appear to exert their effects by imparting different levels of growth autonomy on certain populations of ACF microadenomas and tumors, resulting in their regression or remodelling at the various stages of colon carcinogenesis.

Do changes in fat and ER modulate ACF and tumor development by direct energy deprivation or specific cellular alterations involved in cell growth and differentiation? This question has, in part, been answered by this dissertation. Cell proliferative parameters as measured by mitotic figures and PCNA expression were not consistently affected by fat and ER in carcinogen treated mucosa (Chapters 6-8). Therefore, the resulting phenotypic modulations by dietary fat and energy do not appear to be the result of altered cell proliferative patterns.

The expression of PKC isoforms in carcinogen treated colonic mucosa (at the protein level) was not modulated in any distinct manner by dietary fat and ER, suggesting that the amount of these isozymes do not affect the development of colonic preneoplastic lesions. However, a distinct isozyme expression pattern was observed in colonic tumors, regardless of the diet treatment. Therefore, the expression of these isoforms appears to play a role in tumor progression which is not amenable to changes in diet.

PKC and EGFR-TK activity appeared to be elevated by ER in injected colonic mucosa suggesting that alterations in their activity could be one of the mechanisms by which ER affects preneoplastic development. However, a limitation of this finding

stems from the fact that the homogenate of injected colonic mucosa assayed consisted of a combination of normal and aberrant crypts. Therefore, measurement of enzyme activity, and expression at the protein level, in such colonic homogenates is merely an indication of the state of the whole mucosa, without differentiating between morphologically normal or aberrant crypts. In order to investigate whether the ACF exhibit altered enzyme activity or expression, in relation to normal crypts, a method must be utilized in which ACF are successfully isolated from normal surrounding crypts.

An important contribution of this dissertation was to successfully perform RT-PCR on alcohol fixed ACF in order to study gene expression. By employing RT-PCR it was demonstrated that ACF differ from microadenomas and tumors biologically and that the ability of these lesions to respond or resist growth modulation may reside in their genotypic features. The next step will be to isolate and perform RT-PCR on ACF from different diet treatments exhibiting varying phenotypic characteristics. Such an accomplishment may elucidate the mechanistic actions of dietary fat and energy with respect various enzyme and oncogene expression.

Conclusions.

The findings of the present dissertation support the hypothesis that changes in dietary fat and energy affect specific phenotypic populations of ACF, microadenomas and tumors. In addition, the contention that biological heterogeneity exists among ACF

was further substantiated by the present dissertation. However, the hypothesis that dietary fat and ER affects the development of preneoplastic and neoplastic lesions by direct energy deprivation during specific stages of colon carcinogenesis and/or through alteration of biochemical events involved in cell growth and differentiation was answered in part only. Changes in diet composition affected lesion development at varying stages of colon carcinogenesis albeit more effectively at the early stages of development. Alterations in the expression and activity of enzymes involved in cell growth and differentiation were of limited value in the present research. The method utilized to isolate alcohol fixed ACF provides the impetus to further investigate the cellular mechanism(s) by which diet alters colon carcinogenesis and may serve as the starting point for future investigations towards more comprehensive studies in which the cellular mechanisms involved in the modulation of colon carcinogenesis may become elucidated by incorporating the ACF bioassay and the method utilized to measure genetic aberrations of ACF.

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APPENDIX A

Western blotting Buffers and Gel Recipes (based on the method described by Laemmli, 1970).

All reagents and recipes are from Bio-Rad.

30% Acrylamide/bis mix

87.6 acrylamide (29.2g/100ml)

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

Made to 300 ml with deionized H_2O and stored at 4°C in the dark.

10% Ammonium persulfate

100mg ammonium persulphate in 1ml dieonized H_2O.

10% SDS (sodium dodecyl sulphate)

10 gm SDS dissolved in 90 ml H_2O with gently stirring and brought up to 100

ml volume and stored at room temperature.

Sample Buffer

H_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	0.4 ml
1% (w/v) bromphenol blue	0.4 ml
	8.0 ml

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
(N,N,N',N'-Tetramethylethylenediamine)	5.00 ml

10% Separating Gel

H_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
	10.00 ml

5x Running Buffer

Tris base	9 gm
Glycine	43.2 gm
SDS	3 gm
to 600 ml with deionized H_2O	

Stored at room temperature and diluted to appropriate volume before use.

Transfer Buffer

Tris base	3.03 gm
Glycine	14.4 gm
Methanol	200 ml

The Tris, glycine were mixed in 200 ml methanol and adjusted to a volume of 1000 ml with the addition of deionized H_2O and stored at 4°C.

Coommassie Blue Stain

0.1% Coomassie blue R-250
40% methanol
10% acetic acid
50% deionized H_2O

Stain for ½ hour with gentle rocking.

Destain Solution

40% methanol
10% acetic acid
50% deionized H$_2$O
De-stain with several changes for ½ to 1 hour.

TBST-T

100 mM Tris HCl
0.9% NaCl
0.1% Tween 20

Stored at room temperature, or 4°C for several months.

APPENDIX B

RIPA Buffer for EGFR-TK assay

As detailed in Current Protocols in Molecular Biology, ed. Ausubel et al., 1995 (volume 2, 10.16.8)

With the exception of SDS, all reagents were purchased from Sigma Chemical Co.

1% triton-X 100
0.1%SDS
0.5% sodium deoxycholate
150 mM NaCl
5 mM EDTA
5 mM PMSF
10 μg/ml leupeptin
1 mM Na_3VO_4
5 mM NaPP
20 mM sodium phosphate
1 μl/ml aprotinin
1 mM 1,10 phenanthroline

Stored at 4°C.

APPENDIX C

RT-PCR buffers and Gel recipes based on methods detailed in Current Protocols in Molecular Biology, volume 1 chapter 2.5 (Ausubel et al.(eds), 1995)

All reagents are from Gibco BRL.

5x First Strand PCR Buffer

250 mM Tris HCL (pH 8.3)
375 mM KCL
15 mM MgCl$_2$

10x PCR buffer

200 mM Tris HCL (pH 8.4)
500 mM KCL

10x Loading Buffer

20% Ficoll 400
0.1% SDS
0.25% Bromphenol blue
0.25% Xylene cyanol
 Added 5 μl to 50 μl PCR reaction mix.

10x TBE

Tris HCL   108 gm
Boric Acid   55 gm
0.5M EDTA   40 ml
Bring up to 1 litre volume with deionized H$_2$O.

2% Agarose Gel

H$_2$O   135 ml
Agarose   3 gm
10xTBE   15 ml
Ethidium   4.5 μl
Bromide
(10mg/ml)   _____
                  150 ml

DEPC Solution

0.1% Diethylpyrocarbonate in
       autoclaved H$_2$O

All instruments were immersed and stirred in the DEPC solution for ½ hour prior to autoclaving.

APPENDIX D

Vitamin Mix, AIN-76	g/kg
Thiamin Hcl	0.6
Riboflavin	0.6
Pyridoxine Hcl	0.7
Niacin	3.0
Calcium Pantothenate	1.6
Folic Acid	0.2
Biotin	0.02
Vitamin B_{12}	1.0
Dry Vitamin A Palmitate	0.8
Dry Vitamin E Acetate	10.0
Vitamin D_3 Trituration	0.25
Menadione Sodium Bisulfite Complex	0.15
Sucrose, fine powder	981.08

Mineral Mix, AIN-76	g/kg at 3.5% of diet
Calcium	5.155
Phosphorus	3.984
Potassium	3.602
Sodium	1.019
Chloride	1.571
Sulphur	0.337
Magnesium	0.507
Iodine	0.0002
Iron	0.0351
Copper	0.0056
Manganese	0.0585
Zinc	0.0314