# REGULATION OF THE CELLULAR, MOLECULAR AND MORPHOLOGICAL DETERMINANTS OF COLONIC PRECANCEROUS STAGES BY DIETARY LIPIDS

BY

CAROLYN KATHLEEN GOOD

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements

for the Degree of

Doctorate of Philosophy

Food and Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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# Regulation of the Cellular, Molecular and Morphological Determinants of Colonic Precancerous Stages by Dietary Lipids

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## ABSTRACT

The main objectives of the present dissertation were: a) to evaluate the effect of dietary lipid composition on the stepwise process of colon carcinogenesis; and b) to assess the effects of dietary lipid composition on specific cellular and molecular events associated with cell growth and transformation. These objectives are based on the hypotheses that specific dietary lipids will elicit different effects on the growth of preneoplastic colonic lesions, depending on their stage of development. Furthermore, this effect is mediated via their alteration of the expression of specific proteins involved in cell growth and transformation. A series of studies was conducted to investigate these hypotheses. Briefly, F344 rats were injected with azoxymethane to initiate the carcinogenic process. Intervention with diets containing beef tallow, fish oil, corn oil or corn oil + piroxicam (a cyclooxygenase inhibitor), was begun 12-16 weeks after the final carcinogen injection. At timed intervals (6-8 and 12-16 weeks), animals were terminated and their colons assessed for preneoplastic aberrant crypt foci (ACF) and tumors. Quantification of morphological determinants of carcinogenesis revealed that dietary lipids differ from each other in their ability to affect the growth of preneoplastic lesions at various stages of development. A diet high in fish oil was unable to retard the appearance of advanced ACF and large tumors. The piroxicam-treated group exerted a potent inhibitory effect on tumorigenesis compared to the other diet groups ($P \leq 0.05$). Molecular analysis of tissues was performed by reverse transcription polymerase chain reaction for the

expression of cyclooxygenase (COX1 and COX2), transforming growth factor-α (TGF-α), epidermal growth factor receptor (EGFR) and extracellular-signal regulated kinase-1 (ERK-1). Expression of these target genes in normal colonic mucosa generally differed from tumors. Tumors from different groups exhibited similar morphology, yet displayed molecular heterogeneity depending on the diet treatment. Tumors able to overcome the inhibitory effects of piroxicam had increased mRNA expression of EGFR ($P \leq 0.05$). These findings strengthen the contention that tumors emerge from their progenitor lesions as a result of sequential clonal selection, expansion and adaptation to their biological environment.

## ACKNOWEDGEMENTS

This dissertation would not have been possible without the help and encouragement of many people. First and foremost, I would like to thank my advisor, Dr. Ranjana Prasad Bird. You were the inspiration that lead me down this path. You have been my mentor, friend and constant sounding stone. Thank you for sharing your vision and enthusiasm for research.

I would like to thank my committee members, Dr. Dennis Fitzpatrick, Dr. Geoff Eales and Dr. Michael Eskin. I appreciate the commitment and guidance you have all provided demonstrated throughout my degree.

The excellent technical assistance of Darcy Salo, Brendan McCarthy and Mark Pasetka was an integral part of completing this dissertation. Your willingness to help and good-natured sense of humor always kept me laughing.

A debt of gratitude to all the other "Little Birdies" in the lab, both past and present. You made coming to the lab an enjoyable and memorable experience, and could always be easily persuaded to sit on the radiator and have lunch with me.

I would like to thank my friends, near and far, for supporting and listening to me throughout my graduate program, even though I'm sure you have often wondered what exactly I been doing all this time!

Lastly, to my family, you have given me the support and freedom to pursue and accomplish this goal. For this I will be eternally grateful.

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | arachidonic acid |
| ACF | aberrant crypt foci |
| AIN-76A | American Institute of Nutrition-76 adult diet |
| ANOVA | analysis of variance |
| AOM | azoxymethane |
| COX1 | cyclooxygenase-1 |
| COX2 | cyclooxygenase-2 |
| DHA | docosahexaenoic acid |
| ECL | enhanced chemiluminescence |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| EGFR-TK | epidermal growth factor receptor associated tyrosine kinase |
| EPA | eicosahexaenoic acid |
| ERK-1 | extracellular signal regulated kinase-1 |
| ERK-2 | extracellular signal regulated kinase-2 |
| FAP | familial adenomatous polyposis |
| GAP | GAPase activating protein |
| HFB | high fat beef tallow |
| HFC | high fat corn oil |
| HFF | high fat fish oil |
| HFP | high fat corn oil + piroxicam |
| HNPCC | hereditary nonpolyposis colorectal cancer |
| LFC | low fat corn oil |
| LI | labeling index |
| MA | microadenoma |
| MAPK | mitogen-activated protein kinase |
| NSAIDS | nonsteroidal anti-inflammatory drugs |
| ODC | ornithine decarboxylase |
| PBS | phosphate-buffered saline |
| PCNA | proliferating cell nuclear antigen |
| $PGE_2$ | prostaglandin $E_2$ |
| PGH | prostaglandin H |
| Pi | phosphate ion |
| PKC | protein kinase C |
| $PLA_2$ | phospholipase $A_2$ |
| PLC | phospholipase C |
| p-tyr | phosphorylated tyrosine |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| s.c. | sub cutaneous |
| SD | standard deviation of the mean |
| SDS | sodium dodecyl sulphate |
| TCA | trichloroacetic acid |
| TGF-α | transforming growth factor-alpha |

# CHAPTER 1

# INTRODUCTION

Cancer of the colon is the second leading cause of cancer deaths in North America (Parker et al., 1997). It is now well accepted that environmental factors, including diet, play a role in the carcinogenic process leading to colon tumor formation. As the composition of the modern diet grows increasingly complex, it is difficult to establish a strong association between specific nutrients and cancer incidence based solely on case-control epidemiological studies. The use of animal models has greatly enhanced our ability to study the effects of specific nutrients on colon tumor development under controlled dietary conditions.

Colon carcinogenesis is a multistage process involving the clonal selection and expansion of initiated precancerous cells. A great deal of information exists regarding the *in vitro* growth responses of colon cancer cells, and the nutrient requirements of normal colonic epithelium. However, our understanding of the biology of stages leading up to tumor formation is limited. The concept of cancer prevention involves the regression, inhibition or elimination of precancerous lesions, resulting in the eventual reduction of colon cancer incidence (Bird & Good, in press). In order to achieve this goal, it is essential to have a system that allows the study of the genotypic and phenotypic features of precancerous lesions.

Bird (1987) first identified and postulated that aberrant crypt foci (ACF) found in the colons of carcinogen-treated rodents represented precancerous

colonic lesions. Subsequent studies supporting this contention have lead to the global acceptance and use of the ACF system as a bioassay to study the step-wise development of the disease, and identify potential modulators of colon carcinogenesis. A few studies have questioned the classification of ACF as preneoplastic lesions and value of the ACF system in predicting tumor outcome (Carter et al., 1994; Hardman et al., 1991). More recently, it has become increasingly evident that the structure of the experimental protocol may significantly affect the pattern of ACF growth and development (Bird, 1995). The amount and timing of carcinogen administration, age of experimental animals and period of intervention with specific diets or agents may all impact the final results and conclusions of a given study (Bird, 1995). Such observations strengthen the need to further investigate the role of ACF as a biomarker of colon carcinogenesis, and scrutinize the differences in experimental protocol when comparing studies.

Total intake of dietary fat has been associated with increased tumor incidence in many tissues, including colon (Lewis & Yetley, 1999; Lichtenstein et al., 1998; Reddy, 1994; Woutersen et al., 1999). Recent evidence indicates that not only the amount of fat, but also the specific fatty acid composition of the diet may play a critical roie in colon tumor incidence. Diets high in saturated fat (beef tallow) or polyunsaturated fatty acids (corn oil) have been reported to increase colon tumor formation in animal models (Reddy, 1994; Bird et al., 1996). More recently, the potential inhibitory effect of fish oil on colon tumor incidence, possibly due to its characteristic long chain polyunsaturated fatty acids (PUFA)

docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), has received much attention. However, the effects of these dietary lipids on the growth and progression of precancerous lesions remains relatively unexplored.

Growth factors are known to play an essential role in the homeostatic balance of proliferation and differentiation in normal cells (Tronick & Aaronoson, 1995). However, it is becoming increasingly evident that certain growth factors may initiate the loss of cell cycle regulation associated with tumorigenesis (DiFiore & Kraus, 1992). Transforming growth factor-α (TGF-α) and its receptor (epidermal growth factor receptor, EGFR) are both expressed in colonic tumors, and are essential for the growth of certain colon cancer cell lines (Bauske et al., 1998; Coffey et al., 1987; Zorbas & Yeoman, 1993). Recent evidence has demonstrated that activation of EGFR by TGF-α increases the expression of cyclooxygenase-2 (COX2) in colon cancer cells. Alteration of COX activity and expression has been suggested as a potential mechanism behind the opposing effects of corn oil and fish oil on tumor growth. Whether there exists an association among TGF-α, EGFR and COX in colonic tumor development remains unclear. Furthermore, the expression of these cellular proteins as modulated by the fatty acid composition of the diet has yet to be investigated.

This dissertation evolved from existing data supporting dietary lipids as a modulator of colon tumor incidence, and the lack of data exploring the effect of dietary lipids on the growth of preneoplastic stages of colon carcinogenesis. Employing the ACF system to study the effects of diets varying in fatty acid composition provides a means by which all stages of carcinogenic development

can be evaluated. Such studies may provide valuable insight into specific developmental stages that may be sensitive to growth effects of certain dietary lipids. Assessment of target signaling proteins could provide valuable insight into cellular and molecular changes occurring in the progression to tumor formation.

The hypotheses of the present dissertation are that **(1) specific dietary lipids will elicit different effects on the growth of preneoplastic colonic lesions, depending on their stage of development, and (2) this effect is mediated via their alteration of the expression of specific proteins involved in cell growth and transformation.** Based on these hypotheses, the main objectives of this dissertation were: (a) to evaluate the effect of dietary lipid composition on the stepwise process of colon carcinogenesis; and (b) to assess the effects of dietary lipid composition on specific cellular and molecular events associated with cell growth and transformation.

To accomplish these two broad objectives, five studies were designed to systematically test these hypotheses. The initial study was performed to determine if dietary lipids do indeed modulate proteins involved in cell growth and differentiation. The remaining four studies were designed to explore the main hypothesis in an established animal model harbouring colonic preneoplastic lesions. The specific objectives of each study were to assess the effect of the dietary lipid composition on:

(a) the activation of EGFR-associated tyrosine kinase activity by TGF-α and proliferative indices in non-carcinogen-treated colonic mucosa (Chapter 4);

(b) the growth of precancerous colonic lesions representing different stages of carcinogenesis (Chapter 5);

(c) the growth of ACF and tumor incidence in comparison with the COX inhibitor piroxicam (Chapter 6);

(d) the analysis of specific target signaling proteins in carcinogen-treated colonic mucosa and tumors at the molecular (Chapter 7) and cellular levels (Chapter 8).

# CHAPTER 2

# LITERATURE REVIEW

## *2.0.0 Cancer*

"The disease is not cancer, but the process of carcinogenesis."

Sporn, 1997

Carcinogenesis is a mature, multidisciplinary field of cancer research that has a rich history of accomplishments achieved by various scientific disciplines, most recently molecular biology. Although new investigational techniques provide strong analytical power, major gaps in knowledge remain, relating to the integration of all the individual pieces into a coherent biological framework. Carcinoma is a multi-faceted disease of the whole organism. The ultimate understanding and control of the process of carcinogenesis will require a new fusion at the levels of tissue, organ and organism (Sporn, 1997).

### *2.0.1 Multistage Process of Carcinogenesis*

The development of a fully malignant tumor involves complex interactions among several factors, both exogenous (environmental) and endogenous (genetic, hormonal, immunlogical). Carcinogenesis is a multistage process driven by carcinogen-induced genetic and epigenetic damage in susceptible cells (Harris, 1991). Therefore, cancer can be ultimately described as an aberrancy in normal cell differentiation and survival (Sporn, 1997). Although the continuing process of carcinogenesis comprises multiple interactive factors, it has been broken down into three definable stages: initiation, promotion and progression.

Tumor initiation involves alteration of the cells genome, either by direct

damage from radiation or chemicals, integration of viral genome sequences, or an inherited defect in DNA repair capacity (Bertagnolli, 1997). As opposed to these cells becoming terminally disabled, they gain a selective growth advantage compared to surrounding normal cells. This may be due to a decreased responsiveness to internal and external homeostatic regulators of cell growth and maturation, such as growth factors (Harris, 1991). Other critical targets of DNA damage include the inactivation of tumor-supressor genes, or activation of oncogenes, both of which enhance the probability of neoplastic transformation (Harris, 1991). At least one round of cell division in the presence of the initiating agent or unrepaired DNA damage must occur in order for a cell to be fixed in a initiated state (Pitot, 1993). This requirement may, in part, explain the increased frequency of neoplasms in highly proliferating tissues such as the gastrointestinal tract and skin.

Promotion involves the survival and propagation of initiated cells as compared to, or at the expense of the normal cell population (Harris, 1991). The conversion of an initiated cell to a pre-malignant cell by tumor promoters is a prolonged process, lasting decades in humans (Bertram, 1987). Tumor promoters have distinctively different properties compared to those of carcinogens. Promoters themselves have little carcinogenic activity, and may reduce the time required for tumor development (Weinstien, 1988). Is has also been suggested that the effects of promoters are reversible, indicating that this carcinogenic phase may be the strongest potential target for chemopreventive strategies.

The third and final stage of carcinogenesis is progression, and refers to the

phenotypic changes resulting in the development of malignancy and eventual metastasis (Harris, 1991). Malignant cells possess the ability to travel through the blood or lymph, adhere to a site of distant metastasis and survive in the new tissue environment (Tomlinson et al., 1997). As carcinomas progress, they become increasingly heterogeneous. Cells in advanced metastatic tumors may possess numerous phenotypic and genotypic abnormalities, and these may differ from one cell to another within the carcinoma (Sporn, 1997). The cells of metastasis may have genetic features that are different from those of the primary tumor. Furthermore, perhaps metastatic cells favored to survive the transit to another organ have similar genotypic characteristics (Tomlinson et al., 1997). It is these diverse, yet elusive aspects of tumor cell heterogeneity, which have been major obstacles to the successful treatment and control of advanced tumors (Sporn, 1997).

## 2.1 Colon Carcinogenesis

### *2.1.0 Biology of the Normal Colon*

The basic architecture of the colon is characterized by four distinct layers: the mucosa, submucosa, mucularis externa, and the serosa (Lev, 1990). A single sheet of epithelial cells lines the folds of the mucosa, forming tube-like projections known as the crypts of Lieberkühn (Lev, 1990). Colonic crypts are composed of various cell types, including goblet, absorptive and enteroendocrine cells (Kirkland, 1989). It is hypothesized that all colonic cell species are derived from stem cells located at the base of the crypt (Kirkland, 1989).

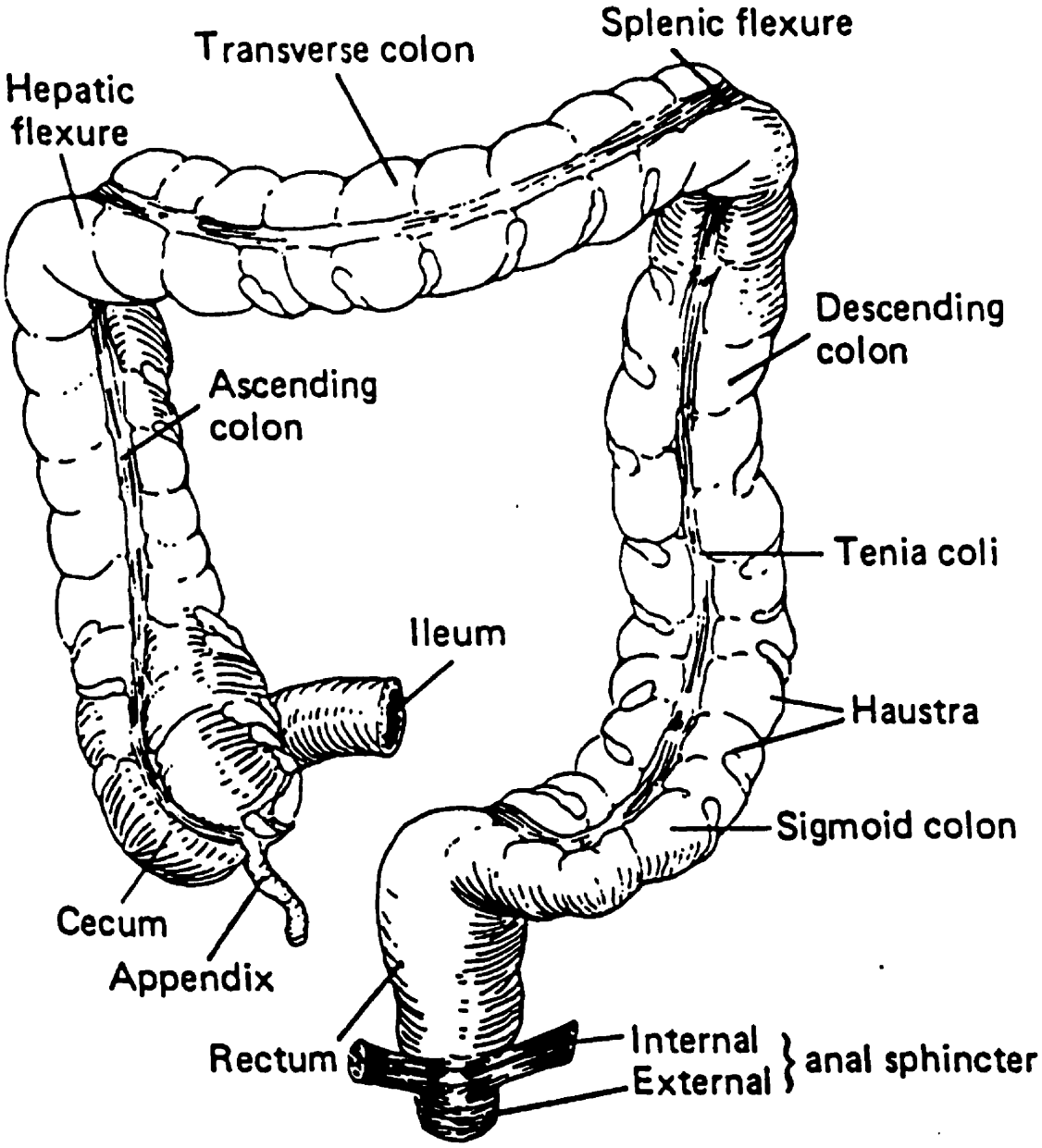
The colonic microenvironment is defined by a tightly regulated balance of cell renewal, maturation and death. The lower two-thirds of the crypt is lined with

immature, dividing cells, and is considered as the proliferative compartment (Simanowski et al., 1989). As cells migrate up the crypt surface, they undergo terminal differentiation and transform into columnar and goblet cells. Upon reaching the top of the crypt, the mature epithelial cells undergo apoptosis, and are eventually sloughed off and shed into the colonic lumen. The crypts are surrounded by a sheath of fibroblasts that migrates upward synchronously with the crypt epithelium (Lev, 1990). The entire mucosal surface is replaced every 4-8 days in humans (Cohen et al., 1989) and every 3-5 days in rats (Maskens and Dujardin-Loitus, 1981). The various mucosal cell populations must achieve four basic functions in order to maintain homeostasis within the crypt: 1) proliferate with proper timing; 2) differentiate in a pattern consistent with normal colonic function; 3) co-operate in a manner such that the rates of proliferation and apoptosis are balanced; 4) repair DNA damage as a result of exposure to potential mutagens (Bertagnolli, 1997). Although crypt cell replication has been extensively studied, relatively little is known pertaining to regeneration of the entire crypt. Although rarely observed, it is thought that formation or repair of damaged colonic crypts occurs in the form of crypt branching (Lev, 1990).

### *2.1.1 Cancer of the Colon*

Cancer of the colon and rectum is the fourth most common cancer in the world. Until recently, half of all colorectal cancers occurred in the rectum and rectosigmoid areas, one quarter in the sigmoid, with the remaining quarter distributed equally among the cecum, the ascending, transverse and descending colon (Figure 2.1). Current evidence now indicates a proximal shift in tumor

Figure 2.1 Physiology of the human colon. Adapted from Ganong, 1993.


Splenic flexure
Transverse colon
Hepatic flexure
Descending colon
Ascending colon
Tenia coli
Ileum
Haustra
Sigmoid colon
Cecum
Appendix
Rectum
Internal
External
anal sphincter

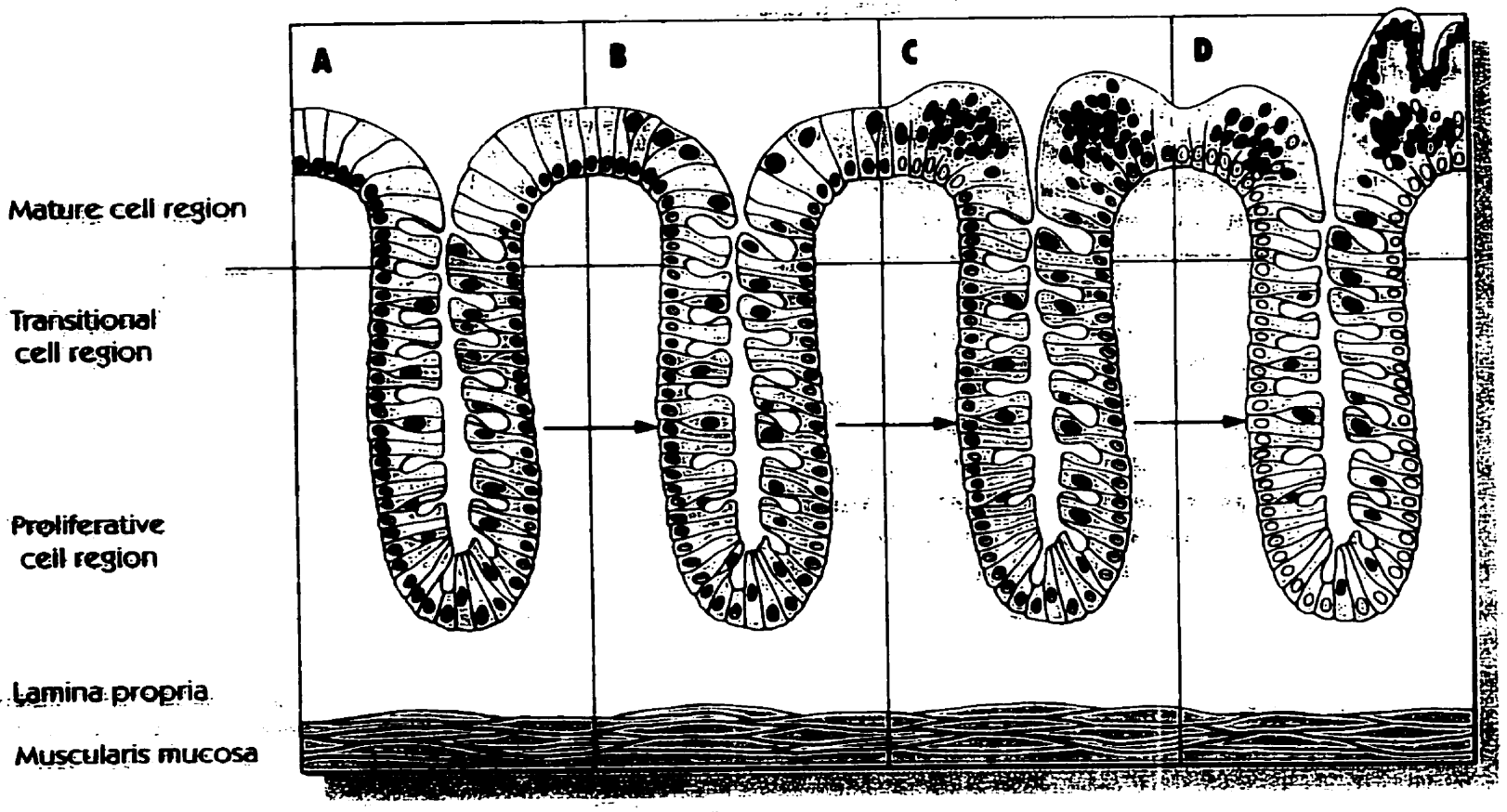
incidence, with an increase in cancer of the ascending colon and cecum (Winawer et al., 1992).

Cancer of the colon is primarily epithelial in nature (Hermanek and Karrer, 1983), and is manifested in a variety of identifiable lesions including; adenocarcinoma; mucinous adenocarcinoma; squamous cell carcinoma; and undifferentiated carcinoma (Morson and Sobin, 1976). It has been reported that approximately 95% of all carcinomas in the colons of humans and animal models are adenocarcinomas (Kumar et al., 1992, Shamsuddin, 1990). The histopathologic features of adenocarcinomas include abnormalities of glandular architecture and epithelial morphology (Lev, 1990). Such adenocarcinomas also retain identifiable colonic crypt structures, confirming development from the mucosa (Fearon, 1995).

### *2.1.2 Histogenesis of Colon Cancer*

Carcinogenesis in the colonic mucosa is characterized by a loss of normal controls and balance of proliferation, differentiation and senescence in the crypt cell population (Bertagnolli et al, 1997). Normal colonic mucosa arises from many stem cells and is therefore polyclonal. Colonic neoplasms are monoclonal in nature suggesting they emerge from a single crypt cell which has experienced an abnormal genetic alteration (Guillem et al, 1995). This genetic event results in the inability of stem cells to repress DNA synthesis during migration from the base to the surface of the crypt (Deschner and Lipkin, 1975). The proliferative zone, normally confined to the lower two-thirds of the crypt, now expands throughout the entire crypt height (Figure 2.2). Loss of proliferative control, or hyperproliferation, is regarded as one of

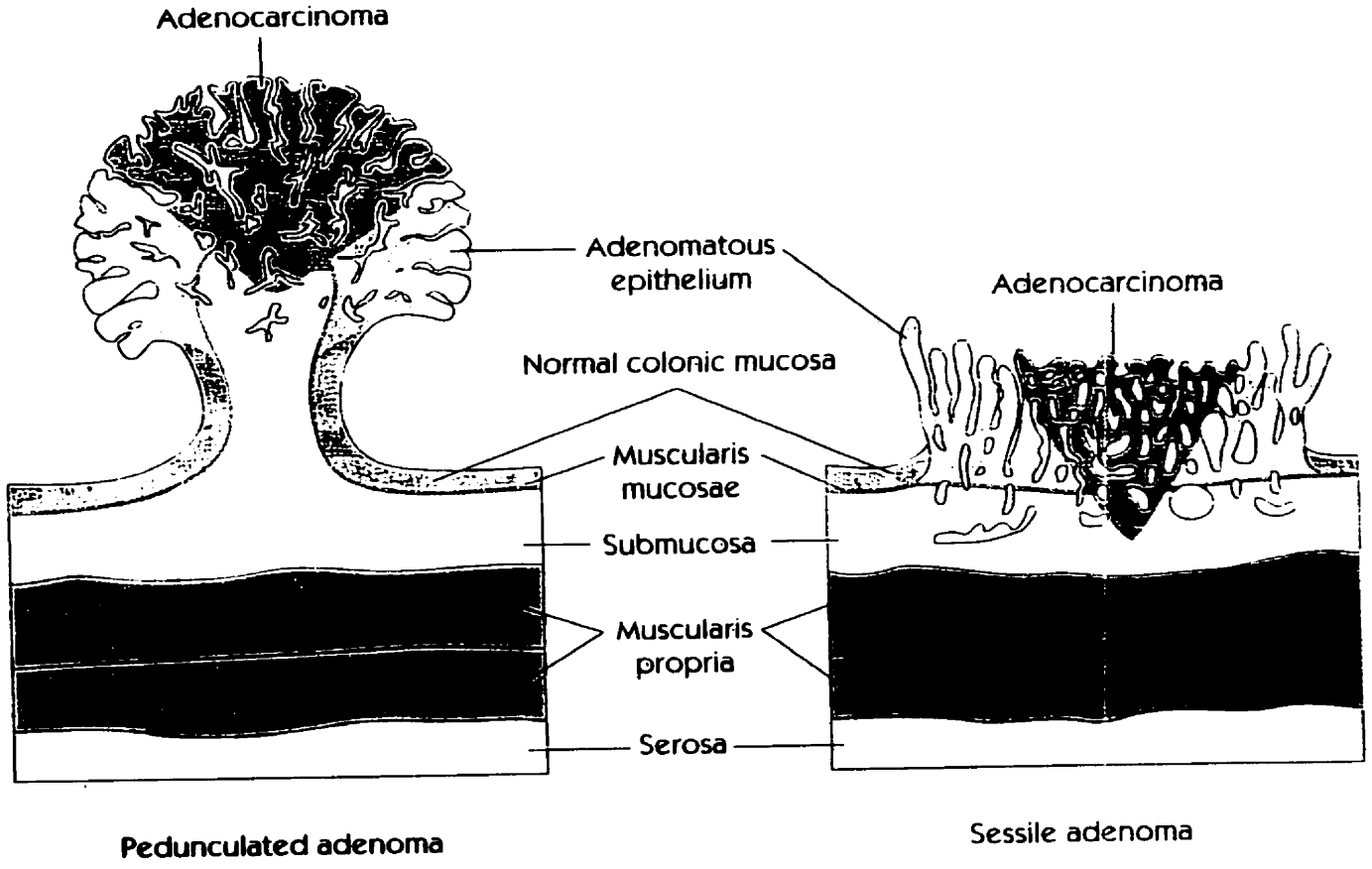
Figure 2.2 This series of diagrams demonstrates: A) normal crypts with proliferation confined to the lower-two thirds; B) expansion of the proliferative zone to the surface of the crypt leading to preneoplasia; C) accumulation of cells at the surface as neoplasia begins; D) initial formation of a polyp. Adapted from Lipkin & Deschner, 1976, and Winawer & Kurtz, 1992.


A
B
C
D
Mature cell region
Transitional cell region
Proliferative cell region
Lamina propria
Muscularis mucosa

the first steps in neoplastic development in the colon (Winawer, 1992). In addition, these cells fail to respond to normal signals for apoptosis and exfoliation, leading to the retention of abnormally dividing cells in the upper surface of the crypt (Deschner and Lipkin, 1975). The transformed epithelium initially spreads along the mucosal surface, eventually invading adjacent crypts replacing pre-existing normal epithelium, a phenomenon known as the "snow-plow" effect (Winawer, 1992). Uncontrolled clonal expansion of the epithelium ultimately leads to the formation of a mass of multiple abnormal crypts that merge to form an adenomatous polyp (Figure 2.3).

Adenomatous polyps, which compose tubular, villous or tubulovillous adenomas, are protrusions of mucosal epithelial tissue that face into the colonic lumen (Lev, 1990). Adenomas have demonstrated malignant potential and are often graded by varying degrees of dysplasia. Progressive dysplasia is characterized by increasingly severe architectural and cytologic abnormalities (Lev, 1990). Dysplasia is categorized as mild, moderate or severe based on the following selected criteria: increased nuclear size; altered nuclear shape; abnormal/disordered mitosis; increased cell crowding; and pattern of glandular branching (Lev, 1990). There is abundant epidemiological, clinical, pathologic and molecular evidence that, with time and accumulation of transformed mutations, colonic adenomas may progress to form adenocarcinomas. This concept is widely known as the "adenoma-carcinoma sequence" or the multistage hypothesis, and was initially proposed by Hill and co-workers (1974). Various lines of evidence support this hypothesis. For example: 1) adenomatous regions are often found in carcinomas; 2) adenomatous

Figure 2.3 Structure of malignant polyps. An adenoma is only considered invasive when malignant cells have penetrated beyond the muscularis mucosa. Adapted from Winawer and Kurtz, 1992.


Adenocarcinoma
Adenomatous epithelium
Normal colonic mucosa
Muscularis mucosae
Submucosa
Muscularis propria
Serosa
Adenocarcinoma
Pedunculated adenoma
Sessile adenoma

coli is almost invariably associated with the development of colon cancer; 3) adenomas and carcinomas have similar distribution along the colon; 4) peak incidence for colonic polyps occurs 5 years prior to the peak incidence of colon cancer; 5) mortality rates are higher in individuals with colonic adenomas; 6) similar dietary factors are epidemiologically related between colonic adenomas and carcinomas (Kumar et al., 1992).

Opposing arguments have been raised from those who do not believe in the adenoma-carcinoma sequence. One theory involves the *de novo* development of colon carcinomas from flat mucosa, without a prior polyp stage (Maskens and Dujardin-Loitus, 1981). Such *de novo* lesions are very rare when compared to the frequency of adenomatous carcinomas, and thus the theory remains on the fringe of scientific acceptance.

### *2.1.3 Proposed genetic model for colon cancer*

Carcinogenesis of the colonic epithelium is likely the result of successive accumulation of multiple genetic mutations, leading to the eventual development of invasive cancer (Bergatolli, 1997). There are two well-established inherited colon cancer syndromes, familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). Both are inherited as autosomal dominant syndromes. Inheritable forms account for approximately 6% of all new cases of colon cancer annually, with the remaining 94% considered as sporadic cancers (Winawer, 1992). It is hypothesized that the seed of genetic damage in both types of colon cancer is planted from two sources: the dominant oncogene and the recessive tumor suppressor gene (Mendelson et al., 1995, Kinzler and

Vogelstein, 1996). Oncogenes originate from normal cellular genes, termed proto-oncogenes, which encode proteins involved in growth factor signal transduction and transcription pathways (Cooper, 1992). Genetic alteration of a proto-oncogene results in a continuous signal, or abnormal signal for cell proliferation or growth (Levine, 1995). One example of an oncogene is *erbB*, which was found to be the truncated form of the epidermal growth factor receptor (EGFR). A mutation in the extracellular domain leads to ligand-independent activation of the receptor, causing its associated tyrosine kinase to be constantly fixed in the active state (Rosen, 1995). In contrast, the products of tumor suppressor genes are used to detect abnormal signals for proliferation or abnormal events in the cell cycle, such as DNA damage (Levine, 1995). In these circumstances, tumor suppressors become negative regulators of proliferation or progression through the cell cycle. Mutations of both alleles (loss of heterozygosity) are required to cause a loss of function in tumor suppressor genes, and can result in: a) a block in the differentiation pathway; b) hyperproliferation; and/or c) a block in normal apoptosis (Boone et al., 1997).

In 1988, Vogelstein and colleagues published a report describing mutational activation of oncogenes coupled with mutation or loss of tumor suppressor genes in colon cancer, and defined their relationship to the adenoma-carcinoma sequence (Vogelstein et al., 1988). A series of genetic changes occurs in the colonic mucosa that leads sequentially to hyperplasia, adenoma, carcinoma *in situ*, and finally invasive cancer. This report has lead to wide acceptance of the multistage hypothesis as the basis for malignant transformation, and provided a genetic perspective to the process of tumor initiation, promotion and progression. The

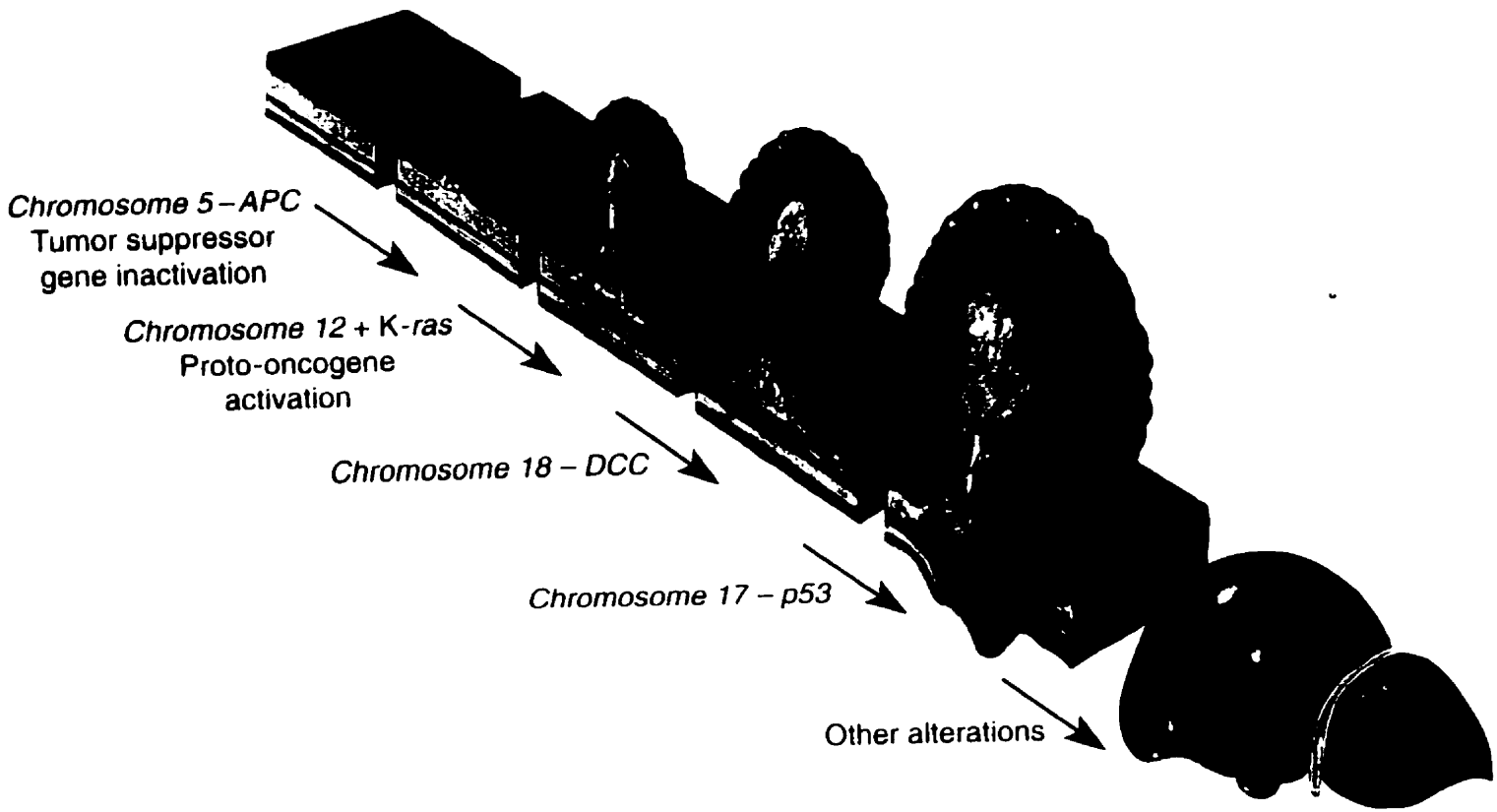
proposed order for the accumulation of mutations in the progression to colon cancer is described in Figure 2.4. Presumably, each mutation confers a selective growth advantage, allowing a clone of cells to overgrow others, ultimately leading to an adenocarcinoma derived from that clone (Kumar et al., 1992). Although these alterations seem to follow a preferred sequence, it is thought to be the overall accumulation of genetic changes more than their ordered sequence that is responsible for cancer development. Mutations associated with colon carcinogenesis are either inherited (as in FAP or HNPCC), or are acquired through interaction with environmental mutagens. However, there is still little understanding of the relationship between dietary and environmental agents associated with increased risk for colon cancer, and the mutational events occurring during neoplastic transformation.

## 2.2 Modulation of Colon Cancer - Experimental Approaches

### *2.2.0 Epidemiological Studies*

Epidemiological observations suggest that environment plays a crucial role in the genesis of colon cancer and may explain the difference in incidence among various countries and societies (Shike, 1995). Colon cancer is the only cancer that exists with almost equal frequency in men and women (Parkin et al., 1992). Occurrence rates vary approximately 20 fold around the world, with the highest rates seen in the developed world and the lowest in India (Parkin et al., 1992). Current evidence also suggests that colon cancer is highly sensitive to changes in the environment. Among immigrants and their descendants, incidence rates rapidly reach those of the host country, sometimes within the migrating generation (Potter,

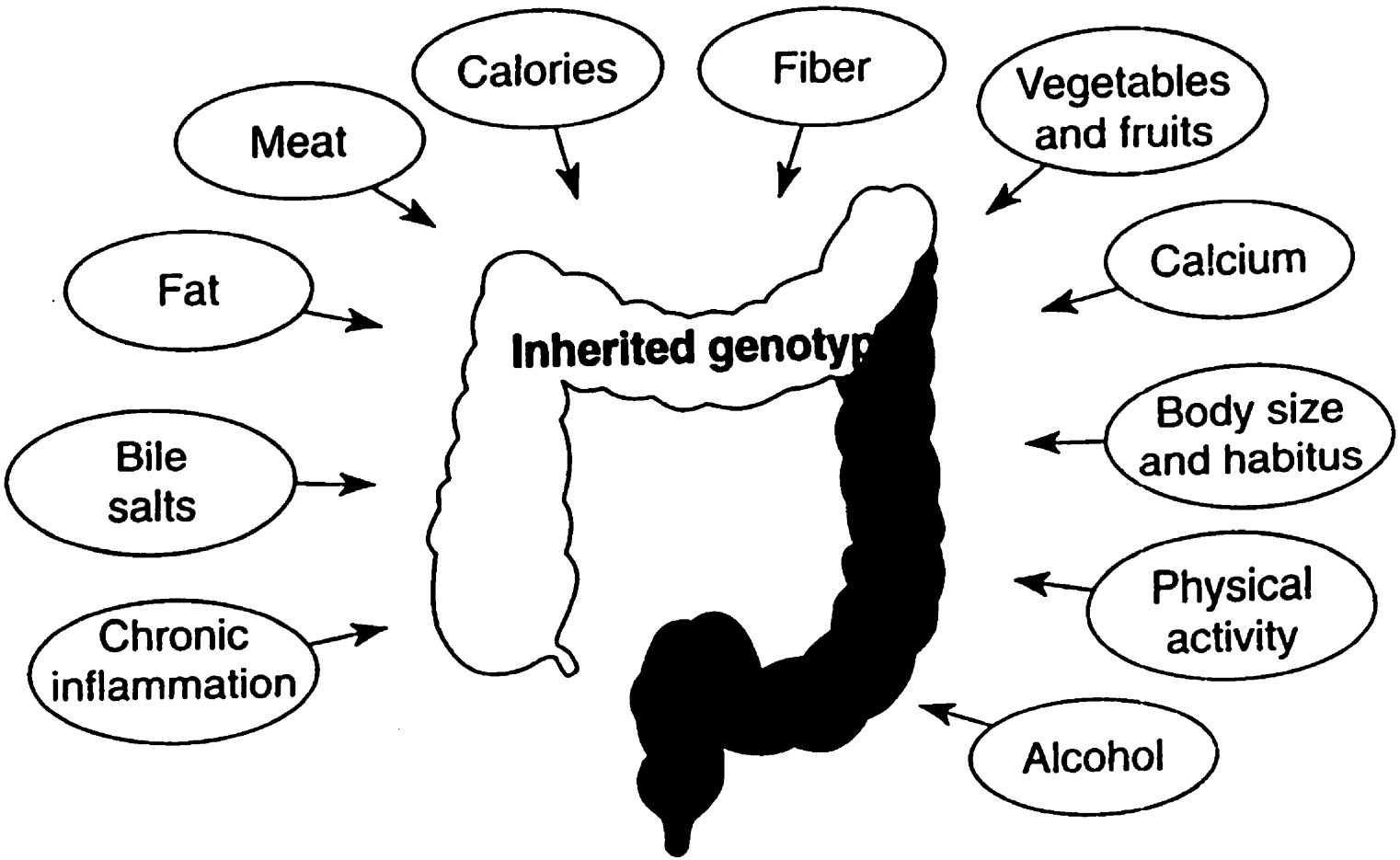
Figure 2.4 A genetic model of the adenoma-carcinoma sequence. Adapted from Gordon and Nivatongs, 1999.


*Chromosome 5 – APC*
Tumor suppressor
gene inactivation
*Chromosome 12* + K-*ras*
Proto-oncogene
activation
*Chromosome 18 – DCC*
*Chromosome 17 – p53*
Other alterations

1999).

Dietary factors are thought to be major etiologic catalysts in the development of colon cancer (Figure 2.5). Determining the relationship between diet and cancer is difficult because of the long interval required for carcinogenesis, as well as multiple confounding interactions between dietary constituents (Bertagnolli et al., 1997). As a result, contrasting epidemiological evidence exists regarding the role of specific nutrients in colon cancer incidence. A recent review of these data indicates that a diet high in meat, saturated fat, total energy and low in fruits and vegetables, fibre, and folate are important promoters of colon cancer (Potter, 1999). Roles of calcium, selenium, and antioxidants in cancer development have been suggested, but with conflicting results (Potter, 1999). Other factors have been implicated in colon cancer risk, such as alcohol intake, physical inactivity and obesity. Evidence suggests that obesity may increase risk of colon cancer, particularly in men (Giovannucci et al., 1995). One lifestyle factor strongly associated with a decreased risk of colon cancer is the use of aspirin or nonsteroidal anti-inflammatory agents (NSAIDS). Regular use of aspirin or NSAIDS in humans correlates with a decrease in colon adenoma and cancer incidence of up to 50% in several large epidemiological studies (Rosenburg et al., 1998; Muscat et al., 1994; Schreinemachers and Everson, 1994, Greenburg et al., 1993). While the data appears promising, widespread use of aspirin and certain NSAIDS is contraindicated in many by side effects of intestinal erosion and increased bleeding tendency (Potter, 1999).

Figure 2.5 Environmental factors that may contribute to the development of colon cancer in high-risk individuals. Adapted from Gordon & Nivatvongs, 1999.


Calories
Fiber
Meat
Vegetables and fruits
Fat
Calcium
Inherited genotyp
Bile salts
Body size and habitus
Physical activity
Chronic inflammation
Alcohol

### *2.2.1 Animal models*

The use of animal models has provided the means to examine and confirm the step-wise evolution of colon cancer. For example, carcinogen-induced colonic neoplasms in rodents were found to histologically mimic those found in humans with regard to the adenoma-carcinoma sequence (Madara et al., 1983). Such model systems also allow the liberty to manipulate and/or control many experimental variables that may influence carcinogenesis, as well as possible mechanisms behind their effects. The type of diet and duration of intervention, timing and frequency of carcinogen administration, strain of animals, and biological end points assessed can all have significant effects on the final outcome of an investigation.

#### *2.2.1.1. Colon carcinogens*

The concept that specific chemicals can cause cancer in experimental animals was first realized in the early 1920s. The induction of colonic tumors was initially reported in rodents that had been fed dibezanthracene or methylcholanthrene (Lorenz and Stuart, 1941). Presently, the two most commonly used chemicals known to induce colonic neoplasms are 1,2-dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM). Both carcinogens are naturally derived from the cycad flower that contains cycasin, a form of methylazoxymethanol (MAM), (Lacquer and Spatz, 1968). Pharmacologic studies demonstrate that these agents require metabolic activation to form the ultimate carcinogen methyldiazonium, an alkylating agent (Greene et al., 1987). The steps required in the activation of DMH and AOM are described in Figure 2.6. Briefly, the activation of DMH shows that it is oxidized to azomethane, which is then converted to AOM.

Figure 2.6 Metabolism of 1,2-dimethyl hydrazine to form azoxymethane in the animal model.

1,2-Dimethylhydrazine $CH_3\text{-}NH\text{-}NH\text{-}CH_3$

↓

Azomethane $CH_3\text{-}N{=}N\text{-}CH_3$

↓

Azoxymethane $CH_3\text{-}\underset{\displaystyle O}{\underset{|}{N}}{=}N\text{-}CH_3$

↓

Methylazoxymethanol $CH_3\text{-}\underset{\displaystyle O}{\underset{|}{N}}\text{-}N\text{-}CH_2OH$

↓

Methyldiazonium $CH_3\text{-}N{\equiv}N$

↓

Carbonium $H_3C{+}N_2$

By process of N-hydroxylation, both AOM and cycasin are converted to MAM, and finally to methyldiazomium (Greene et al., 1987).

Alkylation of both DMH and AOM occurs in the liver, with the activated carcinogen reaching the colon in either the blood or feces. Potter (1999) recently illustrated that the natural evolution for the tube-like design of the colonic crypt was to protect progenitor stem cells from the harmful mutagenic environment of the colonic lumen. Any mutagenic events occurring in the differentiated surface epithelium, which may be in the beginning stages of cell death, would have essentially no effect on the integrity of the crypt cell population. Therefore, the source of the first carcinogenic "hit" in the stem cell population, giving rise to an adenomas is most-likely blood borne rather than luminal (Potter, 1999). Only once the transformed epithelial cells are protruding into the lumen (such as polyp or adenoma) can the colonic contents play a role in neoplastic progression. The importance and pathogenesis of blood-borne compared to luminal carcinogenic agents in the colon is an area of ongoing investigation.

The primary initiating effect of a carcinogen on the colonic epithelial cell is the methylation of DNA. Microscopic adenomas and adenocarcinomas appear in the colons of rats between four and six months after the initial carcinogen treatment (Ward, 1974). Neoplastic lesions induced by either DMH or AOM are similar in terms of development and morphology (Bird et al., 1985). AOM is essentially identical to DMH in terms of colon specificity, but requires fewer activation reactions and is thus closer to the formation of methyldiazonium. It is also chemically stable and easily dissolves in water, giving it theoretical and practical advantages over

DMH (Fiala, 1977).

#### *2.2.1.2. Carcinogen administration*

AOM may be administered either subcutaneously (s.c.) or intraperitoneally. Higher doses of AOM (15 mg/kg) were found to produce a higher number of tumors in the rat distal colon, similar to the incidence of spontaneous colonic tumors in humans (Greene et al, 1987). The age of the animal at the time of carcinogen exposure may also affect tumor outcome, as younger animals are known to be more susceptible to colon cancer development (Bird et al., 1985). Two to three s.c. injections of AOM (15/mg/kg/wk) given to male, weanling rats is a protocol commonly employed by many investigators. Chemically-inducing rodents using AOM is considered to be a simple, reproducible and effective model to study the development of colon carcinogenesis (Banerjee and Quirke, 1998; Greene et al., 1987; Fiala, 1977; Bird et al., 1985).

#### *2.2.1.3. Species and strain differences*

It has been reported that certain strains of rats have a different susceptibility to tumorigenesis under the identical carcinogen protocol (Nauss et al., 1987). Sprague-Dawley and Fisher 344 (F344) are two strains commonly used in the study of colon carcinogenesis. Although Sprague-Dawley rats are considered to be more sensitive to carcinogen treatment, F344 is a more inbred strain (Nauss et al., 1987). Recently, new strains of animals have been developed that show spontaneous colonic neoplasms without the use of a chemical carcinogen. A mouse model with a truncated adenomatous polypolsis coli (APC) gene, referred to as APC1638 mice, develops gastrointestinal adenomas and adenocarcinomas (Fodde et al., 1994).

Alternatively, the Min mouse is a strain with a mutated APC gene that develops intestinal adenomas at a young age, similar to individuals with FAP. Tumors develop much more rapidly in Min compared to APC1638 mice, thus the two models differ in their ability to screen potential colonic chemopreventive agents (Lipkin, 1997).

## 2.3 Biomarkers in the Study of Colon Carcinogenesis

### *2.3.0. Biomarkers*

Tumor markers can be defined as biological indicators of the presence of cancer, whereas, intermediate biomarkers indicate an increased susceptibility to the development of cancer. Biomarkers represent biological factors whose changes parallel the evolution of neoplasms from the initial derangement of cell replication to the malignant carcinoma (Torosian, 1988). The advantage of intermediate biomarkers stems from the shorter time required for their development, thus, their use is more economical in terms of experimental duration and cost (Bird et al., 1989). The confounding realization to the use of biomarkers is that they are merely associated with increased risk for the disease, and do not necessarily represent cancer itself. Therefore, the validity of biomarkers must be carefully scrutinized under a variety of conditions in order to verify their predictive value for cancer development (Bird et al., 1989). Cell proliferation, oncogene expression and changes in cell-signaling pathways are considered to be intermediate biomarkers, and are used to assess cancer risk in colonic tissues (Boone et al., 1992; Risio, 1992). However, the most ideal marker of neoplastic development would be the study of precursor lesions in the colon, since they represent the actual carcinogenic

stage of interest (Bird et al., 1989). It was recently suggested that there should be increased effort in the study of precancerous lesions as a means for cancer prevention (Sporn, 1997).

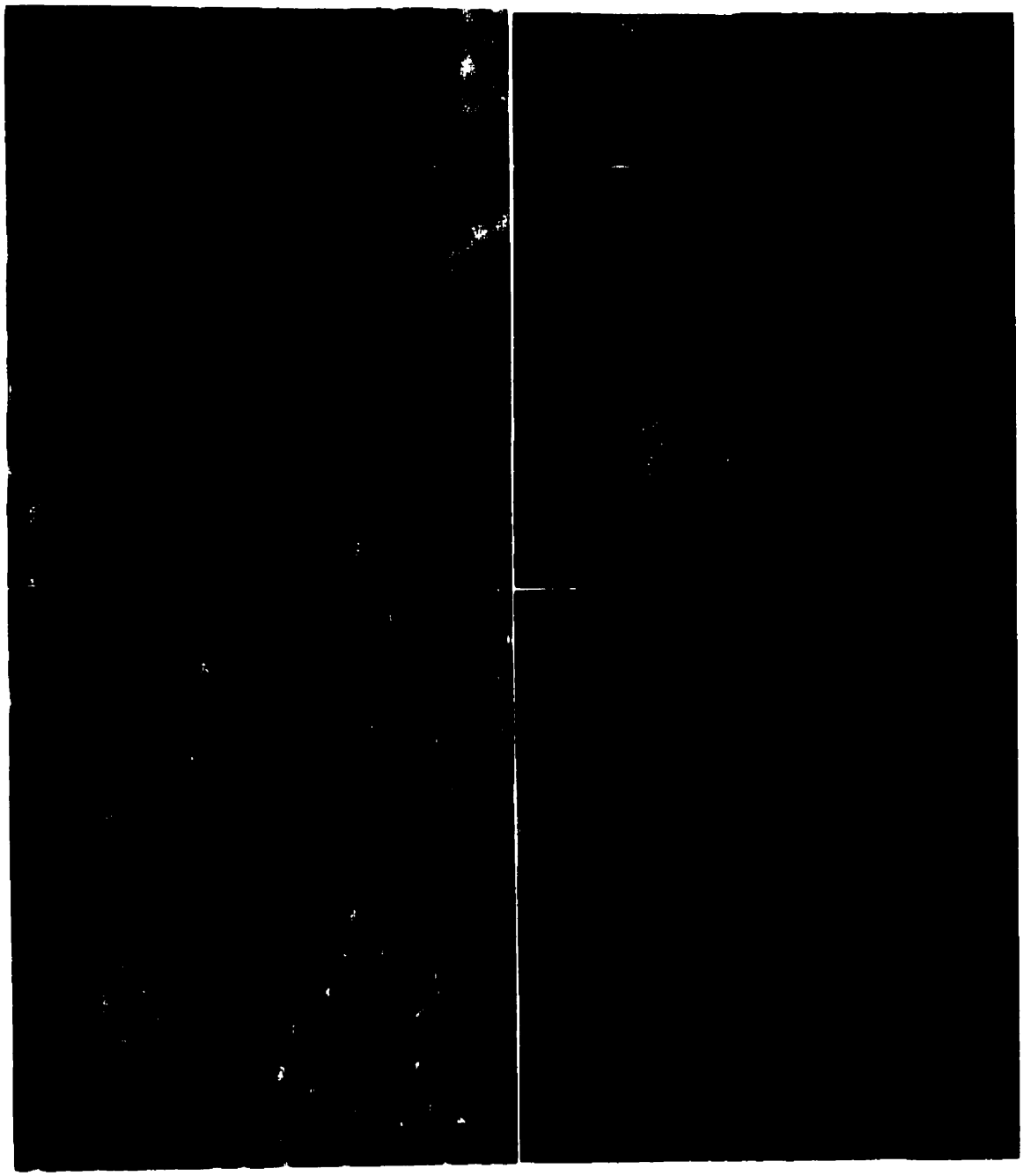
### *2.3.1.0. Aberrant Crypt Foci*

The first identification of precursor lesions in the colon (Bird, 1987) was based on the premise that preneoplastic changes occur in single crypts. These changes must accompany aberrant growth and instability within the crypts, leading to altered crypt morphology (Bird, 1995). Examination of carcinogen-treated mouse colons stained with methylene blue revealed the presence of a single, or cluster of, structurally abnormal crypt(s) (Bird, 1995). These lesions were termed aberrant crypt foci (ACF), and are hypothesized to represent preneoplastic colonic lesions (Figure 2.7). Numerous studies have supported the preneoplastic nature of ACF, and their value as endpoints in the study and identification of modulators of colon carcinogenesis (Bird, 1995). The use of ACF offers many advantages over other intermediate biomarkers, due to the simple, inexpensive and rapid method of their identification and analysis. In addition, enumeration of ACF does require histological preparation or extensive knowledge of cytology (McLellan and Bird, 1988)

### *2.3.1.1. Biological Properties of ACF*

Identifying features of ACF compared to normal surrounding crypts include increased size, thicker epithelial lining, irregular and dilated luminal opening, and increased pericryptal zone (Bird, 1987; Bird, 1998). The initial appearance of ACF in rats and mice has been observed two weeks after a single injection of AOM

Figure 2.7 Methylene blue stained whole mount of colonic mucosa depicting a topographic view (upper panel) and lateral view (lower panel) of an ACF. Note the aberrant crypts have an irregular luminal opening and thicker epithelial lining. The lateral view demonstrates that the ACF is protruded towards the lumen.



(McLellan and Bird, 1988). ACF are also predominantly found in the distal colon, which complements the incidence of colonic tumors in both animals and humans (McLellan and Bird, 1988). The induction of ACF occurs in a dose-related, organ, and species-specific manner by known colon carcinogens (Bird, 1998). A recent study examined the number and location of ACF in rats that had been injected 1 to 4 times with AOM (10 mg/kg/wk, s.c.). All animals were terminated six weeks after the initial carcinogen treatment. The animals that received the greatest carcinogen exposure had the highest number of ACF, most predominantly in the proximal region of the colon (Bird, 1995). This demonstrates that regional differences exist in the colon with respect to carcinogen sensitivity, and that the number of ACF is significantly affected by the frequency of injection and experimental duration after carcinogen exposure (Bird, 1995). Since there is no standard protocol for the induction and use of the ACF system, differences in type, dose, timing and frequency of carcinogen treatment can greatly affect experimental outcome, making comparisons among studies increasingly difficult (Bird, 1998).

The growth and incidence of ACF are quantified by evaluating the following characteristics: 1) the total number of ACF per colon; 2) the size of the focal lesion; and 3) the number of crypts compromising each focal lesion, or crypt multiplicity (McLellan et al., 1991). ACF have been observed to present other phenotypic abnormalities, such as increased proliferative activity (Shpitz et al., 1997), dysplasia (Pretlow et al., 1992a), varying degrees of nuclear atypia (McLellan et al., 1991), and resistance to apoptosis (Magnuson et al., 1994). Sequential histologic analysis revealed that the number of ACF with higher crypt multiplicity increases with time,

and these lesions may represent a more advanced preneoplastic state (Bird, 1995). A recent study assessed ACF for the presence of phosphorylated tyrosine (p-tyr) and transforming growth factor-alpha (TGF-α), which are suggested to be markers of neoplastic change, by immunohistochemistry. While both markers were expressed in normal colonic mucosa, few ACF exhibited immunologically detectable p-tyr (Bird, 1995). Furthermore, the presence of TGF-α was absent in ACF, but was expressed in normal colonic mucosa and tumors (Bird & Good). The potential role of TGF-α in colon carcinogenesis will be discussed in further detail in the upcoming section.

Considering the increase in our understanding of the genetic events involved in colon carcinogenesis, elucidation of the genetic features of ACF is a growing area of interest. ACF exhibit altered expression of K-*ras*, c-*fos*, and p53, genes thought to be critical to colonic tumor development (Stopera et al., 1992 (a); Stopera & Bird, 1993; Stopera et al., 1992(b); Shivapurkar et al., 1994).

#### *2.3.1.2. ACF in human colonic mucosa*

Identification of ACF in humans with colon carcinomas further substantiated the claim that ACF were indeed precursor lesions of colon cancer (Pretlow et al., 1992b). Structural similarities between ACF found in humans with those found in carcinogen-treated rats have been confirmed (Pretlow, 1994). ACF from human colons harbour APC and K-*ras* mutations that are commonly seen in human colonic cancers (Smith et al., 1994). A recent study reported evidence of microsatellite instability in human ACF (Augenlicht et al., 1996). Instability is due to the failure to correct errors that arise during DNA replication, and is also considered as a defect in

mismatch repair. It is known that the inherited development of colonic tumors is partially attributed to defects in mismatch repair, as reflected in HNPCC syndrome (Augenlicht et al., 1996). These observations provide convincing evidence that the ACF system in experimental animals is a valuable and unique tool to study the development of human colon cancer.

#### *2.3.1.3. Value of ACF as a biomarker of colon carcinogenesis*

It has been repeatedly demonstrated that known promoters or inhibitors of colon carcinogenesis also enhance, or inhibit, the number and growth features of ACF (Bird, 1995). It is reasonable to assume that the tumor-modulating effects of various agents may be mediated via different mechanisms, or may target a particular stage of the carcinogenic process. The ACF model is the only bioassay that enables the quantitative assessment of a tumor-modulating agent on the step wise development of colon cancer. The ability to study all stages of carcinogenesis is particularly critical in order to uncover the underlying mechanisms of potential chemopreventive agents, or dietary components. Investigations into the predictive ability of ACF revealed that crypt multiplicity persistently correlated with tumor incidence (Magnuson, et al., 1993; Zhang et al., 1992). However, questions regarding the preneoplastic validity of ACF arose when the number of ACF dramatically decreased, yet tumor incidence dramatically increased in animals exposed to cholic acid, a known tumor promoter (Magnuson & Bird, 1993). It was determined that the reduction in total ACF was the result of cholic acid preferentially promoting a select population of ACF with the highest malignant potential, and eliminating smaller, more primal lesions. This indicates that biological heterogeneity

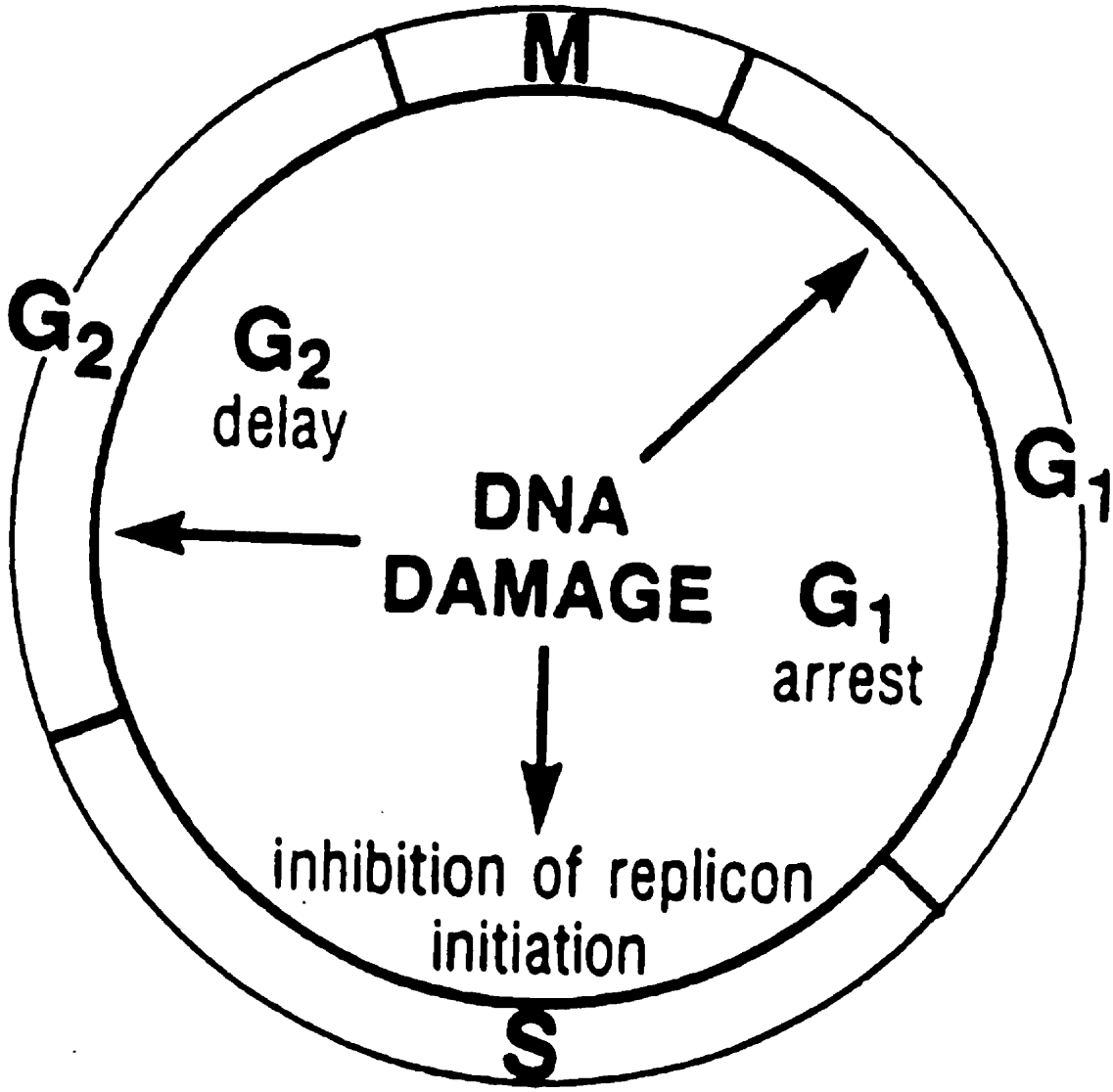
may exist among ACF at different developmental stages.

It is thought that ACF with increasing crypt multiplicity represent advancing preneoplastic states. Based on this concept, it has been suggested that as ACF progress, they attain a certain level of growth autonomy and become more resistant to growth inhibition (Bird et al., 1996). The ACF system can serve as an important instrument for identifying chemopreventive agents capable of targeting preneoplastic lesions with different malignant potentials (Bird & Good, in press). Further investigation into the cellular and molecular features of ACF with various crypt multiplicities may identify events critical to the pathogenesis of colon cancer (Bird, 1995).

#### *2.3.2.0 Cell proliferation*

Proliferation is a central feature of cell biology, as it ensures the anatomical and functional intactness of many tissues (Risio, 1992). Since cancer is a disorder of cell growth and differentiation, cell replication has been extensively investigated as a biomarker of increased cancer susceptibility (Figure 2.8). However, various theories exist regarding the reliability cell proliferation as an indicator of neoplastic potential in select tissues. One stream of thought suggests that an increase in cell proliferation alone, without evidence of other biochemical or genetic alteration, indicates the likelihood of neoplastic development (Ames & Gold, 1990; Cohen & Ellwein, 1990). The contrasting outlook suggests that epithelial carcinomas are not the primary and exclusive result of excess cell proliferation, but the product of multiple interactive factors, including genetic mutation, growth factors and changes in the extracellular environment (Sporn, 1997). The strength in the latter perspective

Figure 2.8 Description of the main stages of cellular replication. Arrows indicate possible areas and effects of DNA damage in the initiation of carcinogenesis. Adapted from Kaufman, 1994.


M
G2
G2
delay
G1
DNA
DAMAGE
G1
arrest
inhibition of replicon
initiation
S

resides in the observation that many normal tissues proliferate more rapidly than cancerous tissues (Sporn, 1997). There is also a wide range of variety in the proliferation characteristics of different regions within the same tissue (Risio, 1992). Furthermore, there has been a lack of association between cell proliferation and cancer occurrence in several tissues, and many genotoxic carcinogens are known to be inhibitors of proliferation (Farber, 1995).

#### *2.3.2.1. Value of cell proliferation as a biomarker of colon cancer*

Colonic epithelium is characterized by active and rapid cell turnover, which is related to the higher proliferative activity in the basal crypt cells (Shptiz et al., 1997). As described previously, modification of cell cycle control leads to the expansion of the proliferative zone to the full length of the crypt. Similar changes have been observed in the colons of patients with sporadic adenomas and familial polyposis (Deschner & Lipkin, 1975; Ponz de Leon et al., 1988). Proliferative activity had also been shown to increase progressively from normal mucosa to adenomas to invasive cancer (Risio, 1992). Conversely, several studies have also reported that changes in cell proliferation do not correlate with end stage tumor development (Cameron et al., 1990; Kingsworth et al.; 1986; Glickman et al., 1987). Moreover, it has been suggested that proliferative changes as a result of diet modification may not be a sound predictor of tumor incidence (Cameron et al., 1990). Use of cell proliferation as an intermediate endpoint to assess the impact of dietary and chemopreventive agents has also yielded conflicting results (Bird & Stamp, 1986; Glickman et al., 1987; Klurfeld et al., 1987; LaFave et al., 1994).

Despite these scientific philosophical differences, cell proliferation remains a

widely used investigative endpoint in carcinogenesis. Athough several immunohistochemical methods exist to evaluate cell proliferation in the colonic mucosa, only two have been well established (Risio, 1992). The first involves the nuclear uptake of pyrimidine analogues of thymidine to label s-phase cells. Bromodeoxyuridine (BrdU) is a thymidine analogue readily taken up by DNA as a alternative to thymidine during DNA synthesis (Risio, 1992).

The second method involves the identification of antigens associated with proliferation, such as proliferating cell nuclear antigen (PCNA). PCNA is an auxiliary protein of DNA polymerase, and plays a critical role in the initiation of proliferation. The antigen is expressed primarily in S-phase cells, but is also found in cells progressing from $G_1$ to S-phase, as well as $G_2$ and M phase cells (Risio, 1992). As a result, PCNA labeling indicies tend to be greater than BrdU labeling indices. Several studies have reported excellent correlations between the measurements of PCNA and BrdU labeling (Risio, 1992; Bird et al., 1989). PCNA has been suggested to be a superior technique compared to BrdU, as it does not require the injection of cytotoxic chemicals prior to sample collection (Risio, 1992). Therefore, PCNA would be the preferred method when both enzymatic and proliferative assessments will be performed on the same sample.

#### *2.3.3.0. Signaling pathways in cell growth and differentiation*

Eukaryotic cells have developed a variety of molecules to "sense" their extracellular environment and intracellular signaling networks, and to transduce this information into the nucleus where it is translated into the appropriate genomic response (Brunet & Pouyssegur, 1997). These complex signaling pathways are in a

large way initiated by the binding of a ligand to its appropriate membrane-bound receptor, activating specific cellular intermediates through a series of phosphorylation/dephosphorylation reactions. The hierarchical organization of this enzymatic cascade allows multiple physiological responses, such as cell-cycle progression, cellular metabolism, and cellular senescence, to be coordinated in a similar manner (Graves et al., 1995). It allows for cell-specific responses, as not all cells express the receptors for, or exhibit similar responses for all ligands. One specific group of proteins that are known to mediate cell-signaling pathways are growth factors. Such factors can influence cell proliferation in positive or negative ways, as well as induce a series of genetic responses in appropriate target cells (Tronick & Aaronson, 1995).

The effect of growth factors depends on a variety of biological ingredients, such as the nature of the growth factor, the cell type and the physiological condition of the responding cell and its environment (Derynck, 1992). Transmission of these biochemical signals to the nucleus leads to the altered expression of a wide variety of genes involved in mitogenic and differentiation responses. Present knowledge suggests the constitutive activation of growth factor signaling pathways affecting these genes contributes to the development and progression in most, if not all, cancers (Tronick & Aaronson, 1995). As mentioned previously, many oncogenes are merely truncated forms of growth factor receptors.

There are four classes of growth factors: 1) growth factors interacting with specific receptors on the cell surface (TGF-α); 2) cell surface hormones (growth hormone); 3) intracellular signal transmitters (ras proteins); 4) nuclear transcription

factors, comprising both initiators (*fos, myc*) and suppressors (p53) of transcription. Interaction of a growth factor with its receptor at the cell surface (class 1) leads to a tight association, allowing growth factors to mediate their message at low nanomolar concentrations (Tronick & Aaronson, 1995). Membrane-associated growth factor receptors contain several discrete domains: the extracellular binding; transmembrane; juxtamembrane; protein tyrosine kinase; and carboxy-tail terminal domains (Ulrich & Schlessinger, 1990). There is substantial evidence that activation of the tyrosine kinase is the initiating trigger of the biochemical events that follow. It is proposed that signals indicating the binding of a ligand to the external domain are transmitted through the transmembrane to induce conformational alterations of the tyrosine kinase, resulting in its activation (Tronick & Aaronson, 1995).

In an alternative model, ligand binding induces the formation of receptor dimers or oligomers. By this latter mechanism, molecular interactions between adjacent cytoplasmic domains lead to activation of the tyrosine kinase by either an intra- or intercellular process (Ulrich & Schlessinger, 1990). The integrity of the tyrosine kinase must be maintained for cell signaling, for any mutation in that region completely inactivates receptor biologic function. The carboxyterminal domain typically contains several other tyrosine residues that are phosphorylated by the activated kinase or other signaling proteins. Phosphorylation of this domain has been postulated to modulate kinase activity and/or the ability of the kinase to interact with its various substrates (Schlessinger & Ulrich, 1992). This demonstrates that growth factor signal transduction pathways are susceptible to positive and negative cross-regulatory inputs from other cellular messaging networks (Graves et al.,

1995).

#### *2.3.3.1. Epidermal Growth Factor Family*

The EGF family consists of EGF, transforming growth factor-α (TGF-α), amphiregulin, betacellulin, heregulin/Neu differentiation factor, and heparin-binding EGF-like growth factor. The EGF receptor (EGFR) is a prototype for a subfamily of structurally related proteins, termed the class 1 receptors. Other members of this family of receptor proteins include erbB2 (also designated as *neu* or *her*), erbB3 and erbB4 (Uribe & Barrett, 1997). Members of the EGF family are classified by a distinct sequence pattern, known as the EGF motif, and high binding affinity for EGFR, with the exception of NDF which binds to erbB4 (Tronick & Aaronson, 1995).

Upon ligand binding to EGFR, there is receptor homodimerization allowing each receptor to phosphorylate key tyrosine residues on the other. The phosphorylated, dimerized EGFR is thereby in an activated state. The process of dimerization also allows for the docking of additional internal proteins to specific tyrosine-phosphorylated sites on the receptor, resulting in their own phosphorylation by the receptor kinase activity (Uribe & Barrett, 1997). These proteins include Phosphoinositol$_3$, Phospholipase Cγ and GAPase activating protein (GAP). Once activated, the receptor now binds the GRB2-Sos adapter protein complex, which in turn leads to the phosphorylation of RAS-GAP. The subsequent steps result in the activation of intermediate signaling kinases such as Raf-1, MEK and extracellular signal regulated kinases-1 and –2 (ERK-1 and -2, also referred to as MAPK).

Figure 2.9 Representation of the epidermal growth factor signaling pathway. Activation of EGFR is linked to nuclear transcription factors through MAP kinase enzymes (also known as ERKs). Adapted from Eling & Glasgow, 1994.

MEK phosphorylation of ERK on its serine/threonine residues results in the phosphorylation of nuclear proto-oncogene nuclear transcription factors such as c-*myc*, c-*jun* and c-*fos* (Figure 2.9). The current view of the EGFR signaling pathway suggests ERK is the protein link leading to the transduction of cytoplasmic, and potentially mitogenic, signals to the nucleus (Eling & Glasgow, 1994).

The vast majority of information regarding the EGF family of peptides is in relation to the functions of EGF and TGF-α. Both share 35% sequence homology, and are known to compete for binding to EGFR with similar affinity. It has been suggested that the two peptides bind differently to EGFR, or cause different conformational changes in the receptor, as there are differences in their biological effects (Oliver et al., 1994). Comparative studies on the effects of EGF and TGF-α have shown that TGF-α is generally more potent in a variety of biological systems (Derynck, 1992). TGF-α was first detected for its ability to induce phenotypic transformation in normal rat kidney cells in culture. In concert with the observation that TGF-α was made only in transformed fibroblasts and not in their normal counterparts, it was predicted that TGF-α could significantly contribute to malignant transformation and tumor development (Derynck, 1992). This is not to say that the growth factor does not play a role in normal physiology. It has been suggested that TGF-α may play a role in development, possibly as a fetal growth factor, since it is highly expression in fetal and epithelial tissues (Massague, 1990). TGF-α has also been implicated in wound healing, and control of acid and other gastric secretions (Uribe & Barrett, 1997).

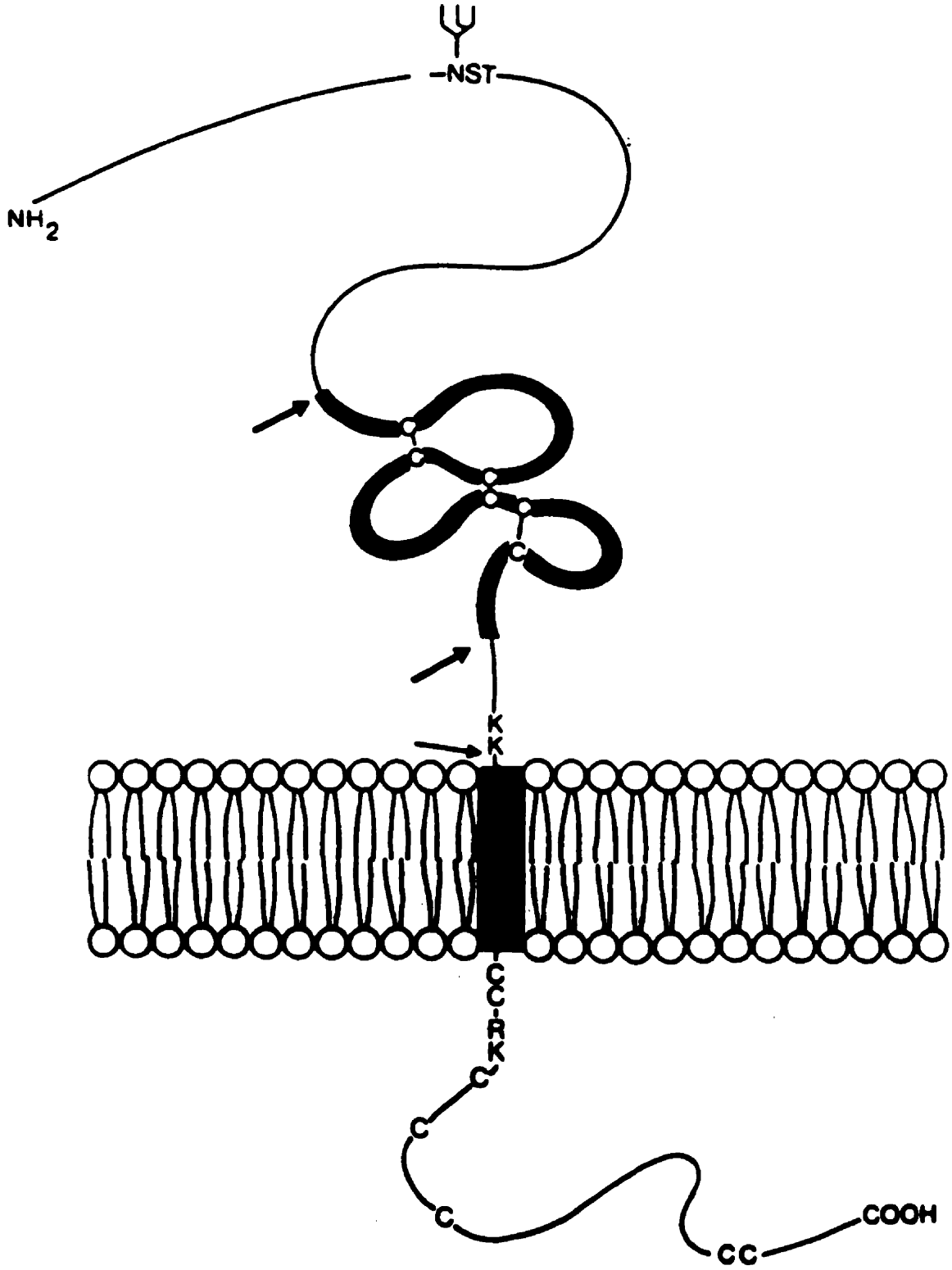
Mature TGF-α is a 50 amino acid peptide formed from its precursor pro-TGF-

α, a glycosylated protein anchored in the cell membrane as described in Figure 2.10 (Teixido et al., 1987). An elastase-like protease cleaves two sites in the middle portion of the extracellular domain of the precursor, resulting in the release of mature TGF-α (Pandiella & Massague, 1991). The identification of TGF-α species with different molecular weights had lead to the suggestion that differential processing of the precursor occurs (Bringman et al., 1987). Various precursor isoforms have been observed at 42 kDa, 40 kDa, 30 kDa, 24 kDa, 20 kDa, 18 kDa and 12 kDa in normal and neoplastic tissue and cell lysates (Tanno & Ogawa, 1994; Bringman et al., 1987; Banerjee et al;, 1998). Recent evidence has indicated that unprocessed remnants of the TGF-α precursor in the cell membrane can also bind to EGFR in neighboring cells, leading to signal transduction (Wong et al., 1989). These findings demonstrate that TGF-α can interact and activate its receptor through juxtacrine, as well as autocrine and paracrine pathways.

#### *2.3.3.2. Role of EGFR and TGF-α in colonic pathophysiology*

The predominant source of TGF-α throughout the gastrointestinal tract is the epithelium, and it has been speculated that the true physical ligand for EGFR in the GI tract is TGF-α rather than EGF (Uribe & Barrett, 1997). Normal colon cells, as well as colon cancer cells produce significant levels of TGF-α and higher molecular weight TGF-α precursors (Zorbas & Yeoman, 1993). Furthermore, coexpression of TGF-α and EGFR, and growth stimulation by TGF-α have been demonstrated in multiple colon cancer cell lines (Zorbas & Yeoman, 1993; Coffey et al., 1987).

Figure 2.10 Schematic diagram of the TGF-α precursor and its processing. Arrows indicate major proteolytic cleavage sites. Adapted from Bringman et al., 1987.


-NST
NH2
K
K
C
C
C
C
C
C
COOH

Increased levels of EGFR and TGF-α were detected in human colorectal tumor samples compared to the normal surrounding mucosa (Messa et al., 1998). Regional differences were also noted, as there was increased expression of TGF-α in the distal compared to the proximal colon, corresponding to the increased incidence of colonic tumors in that area (Messa et al., 1998). The expression of TGF-α and its receptor has also been investigated in preneoplastic tissues, such as ACF. There appeared to be no difference in EGFR expression between ACF and normal appearing colonic crypts (Thorup, 1997).

As mentioned previously, ACF demonstrated a complete lack of TGF-α expression compared to colonic tumors and normal mucosa, yet surprisingly they have been found to express TGF-α mRNA (Bird, 1995; Bird et al., 1997; Bird & Good; Thorup, 1997). This suggests a defect in the translational processing of TGF-α mRNA, or alternatively, the TGF-α protein could be formed and rapidly degraded or secreted from ACF, prohibiting detection by immunohistochemical methods. The lack of TGF-α in ACF also implies that only paracrine and/or juxtacrine stimulation of EGFR occurs, and could perhaps lead to an imbalance in the TGF-α/EGFR relationship and altered growth of ACF (Thorup, 1997).

There has been preliminary evidence to suggest the use of TGF-α and EGFR expression as prognostic indicators in colon cancer patients (McLeod & Murray, 1999). Expression of EGFR is of prognostic significance for a number of solid tumors, including breast and ovarian carcinoma. These findings suggest that normal and malignant colonic epithelial cells do not differ in the existence of growth stimulation of EGFR by TGF-α, but rather in the cellular responses to that

stimulation. As described previously, binding of TGF-α to EGFR activates it tyrosine kinase activity, initiating a cascade of intracellular signals leading to the nucleus.

Changes in TGF-α and EGFR expression in rat colonocytes have been reported as a result of AOM injection (Relan et al., 1995). A 230% increase in colonic mucosal proliferative activity was observed five days after a single injection of AOM (20 mg/kg) was given to five-month old rats. This was accompanied by a 200% rise in EGFR-TK activity, suggesting that activation of EGFR-TK may be a critical event in AOM induction of colonic proliferative processes. Furthermore, this activation is mediated by TGF-α (Relan et al., 1995). This conclusion stems from a comparison of TGF-α and EGF-induced EGFR-TK activity in AOM treated colonocytes that revealed that TGF-α is a more potent stimulator of the receptor (Malecka-Panas et al., 1996). Further studies indicated that incubation with TGF-α resulted in an increase in both EGFR-TK and phospholipase C in rat colonocytes treated with AOM (Malecka-Panas et al., 1998).

These studies indicate a definitive role for TGF-α in stimulation of EGFR-TK in the development of a hyperproliferative state associated with the induction of colorectal neoplasia. However, these were limitations in these studies, as all biochemical measures were performed on colonic mucosa or isolated colonocytes only 5 days after AOM treatment. Therefore, these results are not a reflection of the changes in EGFR-TK stimulation during colonic neoplastic development, but rather an indication of the inflammatory response to carcinogen exposure. The activation and amenability of EGFR-TK activation by TGF-α in preneoplastic colonic mucosa several months after AOM injection has yet to be investigated. Nonetheless, it is

evident that EGFR is involved in the growth and neoplastic alterations in colonic mucosa. Numerous colon cancer cells lines (DiFi, SNU-C1, SNU-C4) are completely dependent on autocrine stimulation of EGFR for growth (Karnes et al., 1998). Treatment of these cells with PD 135053, a selective EGFR-TK inhibitor, induced cell cycle arrest and apoptosis, suggesting it might be an effective treatment of colorectal tumors composed of EGFR-dependent cell types (Karnes et al., 1998).

Normal and neoplastic colonic epithelia do not differ in regards to the existence of a TGF-α/EGFR signaling pathway. However, the nature of responses elicited upon EGFR activation may differ depending on their proliferative vs. differentiated status, or the influence of various environmental factors. As described previously, binding of TGF-α to EGFR activates its intrinsic tyrosine kinase, whereby its signal is transmitted to the nucleus via various intracellular messengers. This communication network is further complicated by the fact that these signaling intermediates not only have the ability to activate nuclear proteins, but are also subject to positive and negative cross-regulatory inputs from other pathways. It has been demonstrated that the ERKs are the terminal recipient of EGFR activation prior to the nucleus. However, few studies have investigated the expression of ERKs in neoplastic tissues. ERKs have been known to activate various associated enzymes, such as cytoplasmic phospholipase $A_2$ ($PLA_2$) and phospholipase C gamma ($PLC\gamma$) (Uribe & Barrett, 1997). Phospholipase enzymes act by liberating arachidonic acid (AA, 20:4 ω-6) from plasma membrane phospholipid stores (Eling and Glasgow, 1994).

Activation of EGFR by TGF-α also stimulates the rapid release of AA due to

the activation of $PLA_2$ and/or PLC (Figure 2.11). Therefore, it appears that EGFR can stimulate the release of AA indirectly through ERKs, or directly via tyrosine phosphorylation of phospholipases by its receptor kinase activity (Hernandez-Sotomayer & Carpenter, 1992; Kast et al., 1993). There are three main enzymes involved in the further metabolism of AA to eicosanoids, including cyclooxygenases, lipoxygenases and epoxygenases (Marks et al., 1999). Eicosanoids are bioactive signaling molecules known to be involved in processes such as inflammation, ovulation, programmed cell death and mitogenesis (DuBois et al., 1996). Conversion of AA to prostanoids involves cyclooxygenase (COX), also known as prostaglandin endoperoxide synthase. At least two COX isoforms have been identified (COX1 and COX2). Both isoforms possess cyclooxygenase and peroxidase activities, which convert AA to prostaglandin $G_2$ ($PGG_2$) and then the intermediate $PGH_2$ (Figure 2.12). The unstable metabolite $PGH_2$ is then converted to an array of prostaglandin species, including prostacyclin ($PGI_2$), thromboxanes and prostaglandin $E_2$ ($PGE_2$). The regulated formation and levels of $PGE_2$ by COX enzymes, and their role in carcinogenesis have been areas of active investigation over the past decade.

#### *2.3.3.3. Role of cyclooxygenases in colon carcinogenesis*

Various lines of evidence support the view that AA and its metabolites may be mediators of tumorigenesis in the colon. Levels of AA in human colonic tumors are increased in the total lipid and phospholipid fractions (Hendrickse et al.,

Figure 2.11 Activation of intracellular signaling pathways and second messenger systems by growth factor receptors through arachidonic acid. Adapted from Nunez, 1997.

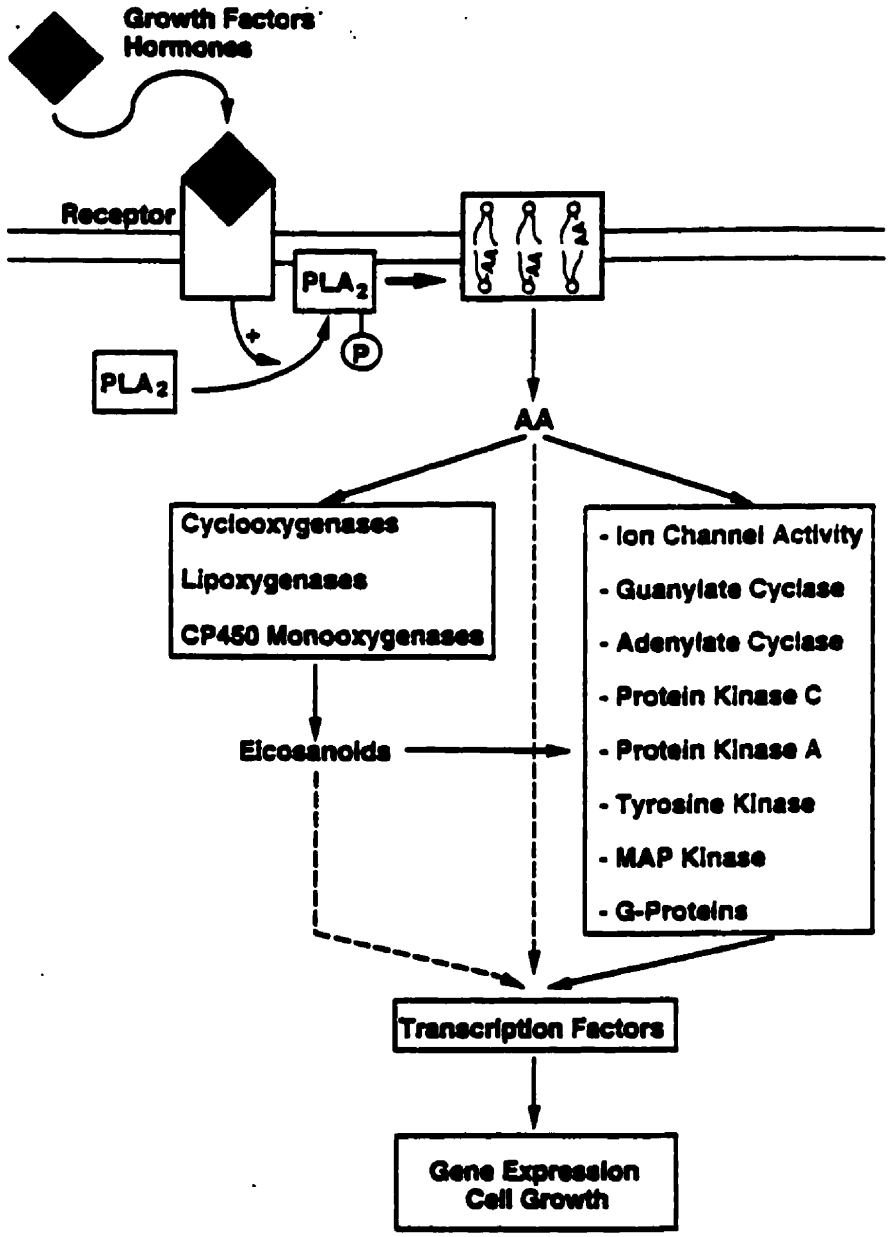
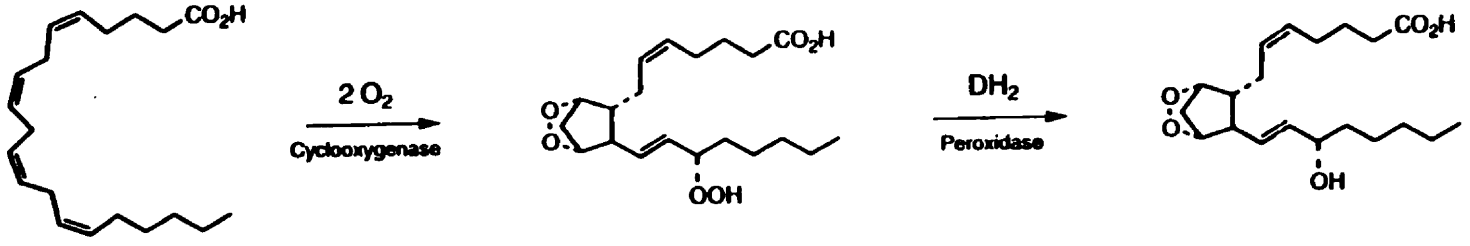

Growth Factors
Hormones
Receptor
PLA2
PLA2
P
AA
Cyclooxygenases
Lipoxygenases
CP450 Monooxygenases
Eicosanoids
- Ion Channel Activity
- Guanylate Cyclase
- Adenylate Cyclase
- Protein Kinase C
- Protein Kinase A
- Tyrosine Kinase
- MAP Kinase
- G-Proteins
Transcription Factors
Gene Expression
Cell Growth

Figure 2.12 Metabolism of arachidonic acid by PGH synthase. Its cyclooxygenase activity converts arachidonic acid to the hydroperoxide $PGG_2$. Next, its peroxidase activity forms $PGH_2$, the precursor for the synthesis of $PGE_2$. Adapted from Marnett, 1994.




Arachidonic Acid

$PGG_2$

$PGH_2$

1994). It also appears that colonic tumors have a greater capacity to release AA from the membrane, as levels of $PLA_2$ were also increased in human colonic tumors compared to normal mucosa (Hendrickse et al., 1995). It is generally thought that $PGE_2$ may play the most significant role in colon carcinogenesis of all the prostaglandins. The concentration of $PGE_2$ is increased in human colonic adenomas and carcinomas, and also carcinogen treated colonic mucosa and tumors from experimental animals (Bennet et al., 1987; Hendrickse et al., 1994; Pugh & Thomas, 1994; Rao et al., 1993; Yamaguchi et al., 1991). The increase in $PGE_2$ in human and rodent colon cancers indicates that colon cancer development is associated with increased COX activity (Gustafson-Svard et al., 1997).

COX exists in two genetically different isoforms, COX1 and COX2, which are likely to represent two partly independent prostaglandin biosynthetic systems (Gustafson-Svard et al., 1997). Both isoforms appear to be expressed at low, but detectable levels in most cell types and tissues (O'Neil & Ford-Hutchinson, 1993; Smith, 1992). In general, COX1 is constitutively expressed, and therefore responsible for the prostaglandin production during basal conditions. A recent study demonstrated that the prostaglandins produced and required for normal functioning of the gastrointestinal tract were derived from COX1 (Kargman et al., 1996). In contrast, COX2 is the inducible form of the enzyme, responsible for increased prostaglandin synthesis in response to mitogens, growth factors and cytokines under inflammatory conditions (Herschman et al., 1997). COX2 has been observed to increase up to 80 fold compared to basal conditions, whereas COX1 has the ability to increase up to 4 fold (Smith et al., 1994). Stimulation of EGFR by TGFα

has been demonstrated to induce COX2 expression and its translocation to the nucleus in both intestinal and colonic cancer cells (Coffey et al., 1997; Tsujii & DuBois, 1995). This is the first direct observation of COX2 induction by the EGFR signaling pathway.

Screening of human colorectal cancers indicated the expression of COX2 mRNA and protein is increased compared to matched normal colonic mucosa (Gustafson-Svard et al., 1996; Kargman et al., 1995; Sano et al., 1995). Furthermore, COX2 mRNA and protein were increased in carcinogen-induced colonic tumors compared to normal–appearing mucosa in rats (DuBois et al, 1996; Gustafson-Svard et al., 1996), and also intestinal tumors in Min mice (Williams et al., 1996). Current evidence indicates that the increased expression of COX2 in human colon cancer originates from neoplastic epithelial cells, as little or no COX2 is expressed by normal colonic epithelium (Kutchera et al., 1996; Sano et al., 1996). Conversely, concentrations of COX1 protein and mRNA seem unchanged or slightly decreased in colonic tumors (DuBois, et al., 1996; Eberhart et al., 1994; Gustafson-Svard et al., 1996; Kutchera et al., 1996; Sano et al., 1995). Altogether, available data from studies on human and experimental colon cancer indicate that COX2 may be the isoform responsible for increased $PGE_2$ production during colon carcinogenesis (Gustafson-Svard et al., 1997).

#### *2.3.3.4. Cyclooxygenase inhibitors in the prevention of colon cancer*

Epidemiologic observations of a reduction in the incidence of colon cancer in regular users of aspirin were first reported in 1988 (Kune et al., 1988). No consistent dose-response relationships were found between aspirin use and

cancers outside the gastrointestinal tract. Various other compounds are recognized as having similar anti-inflammatory and anti-tumorigenic effects as aspirin, commonly known as nonsteroidal anti-inflammatory drugs (NSAIDS). Other lines of evidence exist that indicate a beneficial association between NSAID use and colon cancer risk: FAP patients who took the NSAID sulindac had a significant reduction in adenoma size (Giardiello et al., 1993; Winde et al., 1993); and animal studies of colon carcinogensis have demonstrated that NSAIDS are chemoprotective, causing a reduction in the frequency and number of preneoplastic and neoplastic lesions (Craven & DeRubertis, 1992; Herman et al., 1999; Rao et al., 1995; Reddy et al., 1987).

NSAIDS can be classified based on their binding kinetics with COX enzymes. Certain compounds are simple, competitive inhibitors that compete reversibly with AA for binding to the COX active site, such as ibuprofen, sulindac, and piroxicam. On the other hand, aspirin is a time-dependent, competitive, irreversible inhibitor, known to covalently modify COX (Taketo, 1998). With the knowledge that two isoforms exist, it was determined if certain NSAIDS inhibit one isoform more than the other. Aspirin and ibuprofen are much greater inhibitors of COX1 than COX2; sulindac, piroxicam, and the fatty acid docosahexaenoic acid are equipotent inhibitors of both isoforms; naproxin, Celocoxib, and BF389 are selective COX2 inhibitors (Mitchell et al., 1993).

The majority of studies in experimental models have examined the effects of piroxicam on colon carcinogenesis. Piroxicam was the first available member of a new family of NSAIDS known as the oxicams in 1982 (Lombardino & Wiseman,

1982). Subsequent analyses of piroxicam in rats found it to possess a long-half life, low toxicity, a maximum tolerated dose (MTD) of 500 ppm, and was stable at room temperature (Reddy et al., 1987). A level of 150ppm piroxicam added to the diet was found to decrease tumor incidence when fed at 1 or 13 weeks after carcinogen administration, and was more effective than fellow NSAIDS ibuprofen and ketoprofen (Reddy et al., 1987; Reddy et al., 1992). Piroxicam appears to have a regional effect on colonic tumor incidence, exerting a slightly greater chemopreventive influence on the proximal rather than distal colon (Liu et al., 1995). These findings further illustrate the heterogeneous characteristics and responses to growth modulation that exist within the colon during the carcinogenic process.

The effects of piroxicam on the growth of ACF revealed a reduced number of both primal and advanced preneoplastic lesions compared to control (Morishita et al., 1997; Periera et al., 1996; Wargovitch et al., 1996). It was also suggested that piroxicam was not only able to prevent, but regress the development of ACF, as reported previously with cholic acid feeding (Bird, 1995; Morishita et al., 1997; Pereira et al., 1996). This effect was observed when piroxicam was fed at both 1 and 13 weeks after the initial injection of AOM. It should be noted that all the aforementioned studies added piroxicam to the standard AIN-76 diet composition that contains 5% corn oil as the main lipid source, and is also considered to represent a low-fat control diet. Numerous studies have demonstrated and concluded that a low-fat diet provides a non-stimulatory environment for the growth of ACF and tumors (Bird et al., 1996; Good et al., 1998; Lasko et al., 1999; Reddy, 1992). Animals fed a low-fat diet generally have fewer ACF and tumors compared

to those fed a high-fat diet. It has been suggested that ACF in a low-fat environment may be unstable, thus, more sensitive to dietary changes or chemopreventive agents introduced into that environment. Once preneoplastic lesions reach a certain growth state they exhibit growth autonomy and are increasingly resistant to growth modulating agents (Bird et al., 1996). A high-fat diet appears to promote the growth of ACF, and impels them to reach an autonomous state within a few weeks of feeding. Conversely, ACF require considerably longer achieving this growth state in animals fed a low-fat diet. Therefore, it is unknown whether the inhibitory effects of piroxicam on ACF and tumor incidence would be as significant as currently reported when given in parallel with a high-fat diet.

## 2.4 Dietary Lipids and Carcinogenesis

### *2.4.0 Classification of dietary lipids*

All fatty acids share the similar structure consisting of a hydrocarbon chain terminating with a carboxylic acid group, thus endowing the molecules with a polar hydrophilic end, and a non-polar hydrophobic end (Hunt & Groff, 1990). The length of the fatty acid chain ranges from four to 24 carbons atoms, and may be saturated, monounsaturated or polyunsaturated, referring to hydrogen saturation of the carbon chain. The main classes of fatty acids are described in Table 2.1. Unsaturated fatty acids may possess one to four double bonds, which may exist in the *cis* or *trans* configuration. Most naturally occurring polyunsaturated fatty acids (PUFA) are found in the *cis* configuration, while *trans* fatty acids are often found in processed and hydrogenated fats and oils (Hunt & Groff, 1990). A notation has been established to denote the chain length of the fatty acids, as well as the number and

**Table 2.1 Names and Chemical Structures of Common Saturated and Unsaturated Fatty Acids**

| Notation | Common Name | Formula |
|---|---|---|
| **Saturated Fatty Acids** | | |
| 14:0 | Myristic acid | $CH_3$-$(CH_2)_{12}$-COOH |
| 16:0 | Palmitic acid | $CH_3$-$(CH_2)_{14}$-COOH |
| 18:0 | Stearic acid | $CH_3$-$(CH_2)_{16}$-COOH |
| **Unsaturated Fatty Acids** | | |
| 18:2 ω-6 | Linoleic acid | $CH_3$-$(CH_2)_4$-CH=CH-$CH_2$-CH=CH-$(CH_2)_7$-COOH |
| 18:3 ω-3 | Linolenic acid | $CH_3$-$(CH_2$-CH=CH$)_3$-$(CH_2)_7$-COOH |
| 20:4 ω-6 | Arachidonic acid | $CH_3$-$(CH_2)_3$-$(CH_2$-CH=CH$)_4$-$(CH_2)_3$-COOH |
| 20:5 ω-3 | Eicosapentaenoic acid | $CH_3$-$(CH_2$-CH=CH$)_5$-$(CH_2)_3$-COOH |
| 22:6 ω-3 | Docosahexaenoic acid | $CH_3$-$(CH_2$-CH=CH$)_6$-$(CH_2)_2$-COOH |

position of any double bonds that may be present. The first number represents the number of carbon atoms, followed by the number of double bonds, and finally the location of the double-bond nearest the methyl (omega, ω or "n") end of the chain. For example, the notation 18:2 ω-6 describes linoleic acid.

The ω-3 and ω-6 PUFAs are considered to be essential fatty acids as they cannot by synthesized by the body, and therefore must be obtained from the diet. Their essentiality is due to the fact that most vertebrate species lack the enzyme required to incorporate a double-bond beyond the 9$^{th}$ (n-9) carbon in the chain (Hunt & Groff, 1990). Although the body cannot make these essential fatty acids directly, they act as substrates for the formation of long-chain fatty acids through various elongation and desaturation reactions. Linoleic acid is the precursor to arachidonic acid (AA, 20:4 ω-6), and linolenic acid (18:3 ω-3) is the precursor to eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3). These long-chain fatty acids are not essential as they can be obtained through dietary sources. Competition between ω-3 and ω-6 fatty acids occurs at the level of desaturation and elongation. The main enzyme involved is the Δ6 desaturase, which has greater affinity for linolenic compared to linoleic acid. Thus, an abundance of linolenic acid can effectively decrease the formation of AA, and increase the formation of EPA and DHA (Hunt & Groff, 1990).

### *2.4.1. Physiological properties of dietary lipids*

Dietary lipids are essential components of numerous cellular systems. This concept is illustrated by the influence of dietary lipids on the onset and progression of diseases such as heart disease, diabetes and cancer. Most biological

membranes contain a variety of polar lipids, and as many as 40 different fatty acids may be incorporated into the phospholipid molecule (Clandinin et al., 1991). Alterations in membrane fatty acid composition have been related to dietary sources of lipids. This occurs as a consequence of the essential nature of linoleic and linolenic acid, as *de novo* membrane phospholipid synthesis allows for the incorporation of new fatty acids from dietary origin (Clandinin et al., 1985). Initial physiological responses to changes in dietary fat intake occur in the gastrointestinal tract at the level of the enterocyte brush border (Robblee & Bird, 1994). The relative availability of saturated and unsaturated fatty acids for brush border phospholipid synthesis is also determined by the activity of elongation and desaturation enzymes. A described previously, the activity of the Δ6 desaturase is the rate-limiting step in the conversion of linoleic acid to AA. It has been shown that desaturase activity responds to the composition of dietary fatty acid intake, thus affecting the type and amount of fatty acid incorporation into the enterocyte membrane (Clandinin et al., 1991).

The membrane lipid bilayer also contains a variety of intrinsic and extrinsic proteins. It is thought that the lateral mobility of such proteins within the bilayer is dependent on the membrane lipid composition. Several covalent and non-covalent forces are involved in the interaction of membrane lipids and proteins, and has lead to the concept that a protein requires a specific lipid microenvironment in order to function, and may possess increased affinity for those lipids (Clandinin et al., 1994). Alterations in membrane lipid composition may therefore affect the function of membrane proteins such as receptors and other membrane-associated enzymes. It

has been repeatedly demonstrated that membrane fatty acids provide an integral role in second messenger signaling pathways associated with receptor activation (activation of protein kinase C by diacylglycerol), and production of other bioactive intermediates (eicosanoids) (LaFave et al., 1994; Robblee & Bird, 1994).

Immunopurified EGFR has shown to be activated in vitro by oxidized LDL and 4-HNE, a major lipid peroxidation product (Suc et al., 1998). It was recently demonstrated that the addition of linoleic acid and AA to fibroblasts increased both EGFR-TK and MAPK activity (Eling et al., 1997). Comparatively, it was also found that increased EGFR activation increased linoleic acid metabolism and eicosanoid production, resulting in increased cell proliferation (Eling & Glasgow, 1994). These findings suggest that linoleic acid metabolism plays a central role in the transduction of the EGFR mitogenic signal to the nucleus, and various lipid species can influence the initial activation of EGFR.

The notion that specific polyunsaturated fatty acids may regulate the expression of specific genes is a rather recent discovery. There exist two schools of thought as to the mechanisms of fatty acids induced gene expression. One involves an indirect route through the activation of second messengers as mentioned briefly. Addition of exogenous AA to 3T3 fibroblasts increased expression of the nuclear transcription factors, such as the proto-oncogenes c-*fos*, c-*myc* and c-*jun* (Sellmayer et al., 1997). AA stimulated expression of these transcription factors through its production of $PGE_2$. In contrast, there was no accumulation of c-*fos* after the addition of EPA or DHA, as they antogonized the effects of AA and decreased the synthesis of $PGE_2$ (Sellmayer et al., 1997). A contrasting model suggests that the

Δ6 desaturation of linoleic and linolenic acid is a prerequisite step in the generation of a bioactive regulator for gene transcription. The long chain PUFAs are then directly transferred to the nucleus, where they function as ligands or modifiers of nuclear fatty acid-receptor proteins. Following the PUFA-dependent modification of this binding protein, it interacts with a *cis*-acting element in the target gene that governs gene transcription (Clarke & Jump, 1994). This theory has gained merit since the recent observation that the cyclooxygenase metabolite 15-deoxy-12,14-$PGJ_2$ directly binds to and activates the transcription factor peroxisome proliferator-activated receptor γ (Kliewer et al., 1995). In summary, dietary lipids can affect cellular responses by modulating plasma membrane fatty acid composition, altering the activity of membrane receptors and other signaling intermediates leading to altered gene expression. Furthermore, recent evidence alludes to the ability of PUFA to induce gene transcription by interacting directly with fatty acid binding element on nuclear transcription factors.

### *2.4.2. Epidemiological studies*

A number of epidemiological studies have demonstrated the influence of environment, such as dietary factors in the etiology of colonic cancer. Although few relationships between diet and cancer incidence are definitively established, recurring trends from epidemiological evidence and migrant studies indicate dietary fat as a potential risk factor for colon cancer. A high correlation between national per capita disappearance of fat and national rates of colon cancer led to the hypothesis that consumption of fat, especially from animal sources, increased risk for colon cancer (Giovannucci & Goldin, 1997). Firstly, in order to give proper

consideration to these findings, one must consider that a high total fat intake is often proportional with a high-energy intake. Total caloric intake alone increases risk for colon cancer. The overall evidence from a recent meta-analysis of 13 case control studies suggests that higher intake of all energy sources increases risk of colon cancer ie: replacing a equivalent amount of energy from carbohydrates with protein would not reduce one's risk of colon cancer (Howe et al., 1997). Therefore, the overall hypothesis that total fat consumption in the diet, independent of total energy intake, increases colon cancer risk is not supported by the results of these studies.

In case-control studies that did show a positive association between fat and colon cancer risk, the relation was usually attributable to fat from animal sources or saturated fat (Potter et al., 1993; Slattery et al., 1997; Willet et al., 1990). However, three of four extensive cohort studies with a comprehensive assessment of diet did not find an association between total, animal, vegetable, or polyunsaturated fat and risk of colon cancer (Bostick et al., 1994; Giovannucci et al., 1994;Willet et al., 1990). It has been argued that as specific fatty acids possess different biological activities, the independent contributions of certain fatty acids may be significant, and under valued, in colon cancer risk. The protective role of marine long chain ω-3 fatty acids (EPA, DHA) in heart disease sparked interest in the possible chemoprotective characteristics of these dietary fatty acids in colon cancer.

Certain case-control studies have found an inverse, but not significant, correlation between fish ω-3 fatty acids and colon cancer incidence (Caygill et al., 1996; Hurtsing et al., 1990; Willet et al., 1990). Sequential phospholipid analysis of benign adenomas to invasive colon tumors revealed decreasing levels of EPA with

increasing disease severity (Fernandes-Banares et al., 1996). Fish oil supplementation (up to 9 g/day) has been reported to decrease colonic mucosal proliferation in both healthy individuals and patients with colonic adenomas (Anti et al., 1994; Bartram et al., 1993; Huang et al., 1996). However, the impact of fish oil supplementation was only significant in mucosa already displaying hyperproliferative characteristics, and when continued for at least 6 months. It is interesting to note that there was a significant increase in EPA and decrease in linoleic acid and AA in colonic mucosal lipids after 6 months of fish oil supplementation (Anti et al., 1994), reaffirming the influence of dietary lipids on membrane fatty acid composition. Although these data indicate that fish ω3 fatty acids may appear to be a promising chemopreventive agent, as of yet there are no intervention studies with fish oil utilizing adenoma and tumor incidence as an end point.

### *2.4.3. Animal studies*

#### *2.4.3.0. Tumorigenesis studies*

Interpretation of earlier animal model studies on dietary fat and colon cancer were confounded by the use of formulated diets with different caloric density and levels of other essential nutrients eg: low-fat vs. high-fat diet. It is also known that animals will adjust their food intake so that similar energy intake is maintained even with diets containing different energy density (Reddy et al., 1985). Therefore, a diet lower in energy value (low-fat) will be consumed at a greater rate, along with the amount of added nutrients, than a diet higher in energy value (high-fat). Experimental diets used in present tumorigenesis studies have been adjusted to ensure that animals receiving a high-fat diet will also receive an intake of protein,

vitamins and minerals comparable to a low-fat diet (Reddy, 1994).

Much of the initial interest of the effects of dietary lipids on tumor incidence in animal models stems from the observations that, irrespective of carcinogen used, diets high in beef fat and corn oil had a greater tumor enhancing effect than a low-fat diet (see Reddy, 1994 for review). Further investigation of other dietary lipids revealed that diets high in corn and safflower oil were tumor promoting compared to their low-fat counterparts, whereas, diets high in olive and coconut oil and trans fatty acids had no tumor enhancing effects (Reddy & Maeura, 1984). Reports that diets high in menhaden/fish oil inhibited carcinogen-induced mammary cancer in rats (Karmali et al., 1984), sparked interest in its potential preventive effects in colonic tumor development.

An initial study by Nigro and colleagues (1986) demonstrated no significant decrease in rat colonic tumor incidence by fish oil, although it was only supplied as 3% of the total diet lipid content. Subsequent studies revealed a tumor inhibiting effect of high fat diets containing at least 17% fish oil compared to a high corn oil diet (Reddy & Sugie, 1988). Since the specific long chain ω-3 fatty acids were thought to be responsible for the cancer protective ability of fish oil, the effects of EPA and DHA were respectively investigated in carcinogen-induced colon cancer. It was shown that both EPA and DHA did decrease colonic tumor incidence in AOM-treated rats compared to a standard low-fat corn oil diet (Minoura et al., 1988; Takahashi et al., 1997). However, it should be noted that the animals were given 1 ml of DHA by gastric intubation 5 times per week (Takahashi et al., 1997), compared to EPA that was given chronically through the diet as done in previous studies

(Minoura et al., 1988).

Difficulties in comparing results from one study to another often arise due to differences in experimental design, carcinogen treatment, diet composition and length of dietary intervention. The experimental diets are usually given prior to, during, or immediately following carcinogen treatment and continue until end stage tumor development. There exists the possibility that dietary nutrients may interfere with carcinogen metabolism, thereby reducing tumor incidence by decreasing the number of initiated cells. However, one could mistakenly draw the conclusion that the experimental diet is tumor inhibiting, when it is merely decreasing the effectiveness of the carcinogen. Due to these confounding factors, it still remains unclear as to the specific stage(s) at which dietary lipids exert their most prominent tumor enhancing or inhibiting effects.

#### *2.4.3.1. Studies using the ACF system*

The effect of dietary lipids on the growth of ACF has not been as well defined as in tumor incidence studies. The growth promoting ability of diets high in corn oil or beef tallow on ACF has been reported by several investigators (Bird et al., 1996; Bird & Lafave, 1995; Lasko et al., 1999; McLellan & Bird, 1988; Robblee & Bird, 1994; Shivapurkar et al., 1992), and supports the tumor promoting ability of these diets. Although a high-fat olive oil diet was though to be anti-tumor promoting, it had effects similar to a high-fat corn oil diet on the growth of ACF induced by 20 mg/AOM (Bird & Lafave, 1995). This was a relatively short-term study (8 weeks), and it is possible that olive oil exerts its inhibitory effects on the later stages of colon carcinogenesis that were not assessed. The effects of DHA on ACF development

revealed that the fatty acid did decrease the total number of ACF per colon, and also the crypt multiplicity compared to control (Takahashi et al., 1994). Once again, DHA (0.7 ml) was given twice weekly through gastric intubation, making interpretation among studies difficult. Simultaneous investigation of both promoting and inhibiting fatty acids on the growth of ACF under equivalent experimental conditions has yet to be done.

### *2.4.4. Effect of lipids on signaling pathways in colonic mucosa*

One mechanism proposed to account for the tumor modulating ability of dietary lipids is their influence on signal transduction pathways. The cellular targets influenced by dietary factors in cancer promotion and progression are illustrated in Figures 2.13 and 2.14. One example is PKC, which has been implicated in colon carcinogenesis through its role in signaling pathways leading to cell proliferation. A high beef tallow diet has been shown to increase membrane-associated (PKC) activity compared to a low-fat diet in carcinogen-treated rat colonic mucosa (LaFave et al., 1994). It was also demonstrated that a high beef tallow diet altered the fatty acid profile of the phosphotidylethanolamine fraction of the colonic mucosa by increasing the amount of AA, a known activator of PKC (LaFave et al., 1994). Other signaling enzymes that are modulated by the dietary fatty acid composition include ornithine decarboxylase (ODC), a member of the polyamine biosynthetic pathway, and tyrosine protein kinase. A high fat corn oil diet has been reported to induce both ODC and tyrosine kinase activity in carcinogen-treated rat colonic mucosa compared to other high fat diets containing olive oil or fish oil (Reddy, 1994). As

Figure 2.13 Influence of dietary factors on growth factor production associated with abnormal cellular replication and eventual formation of precancerous lesions. Adapted from the World Cancer Research Fund, 1997.

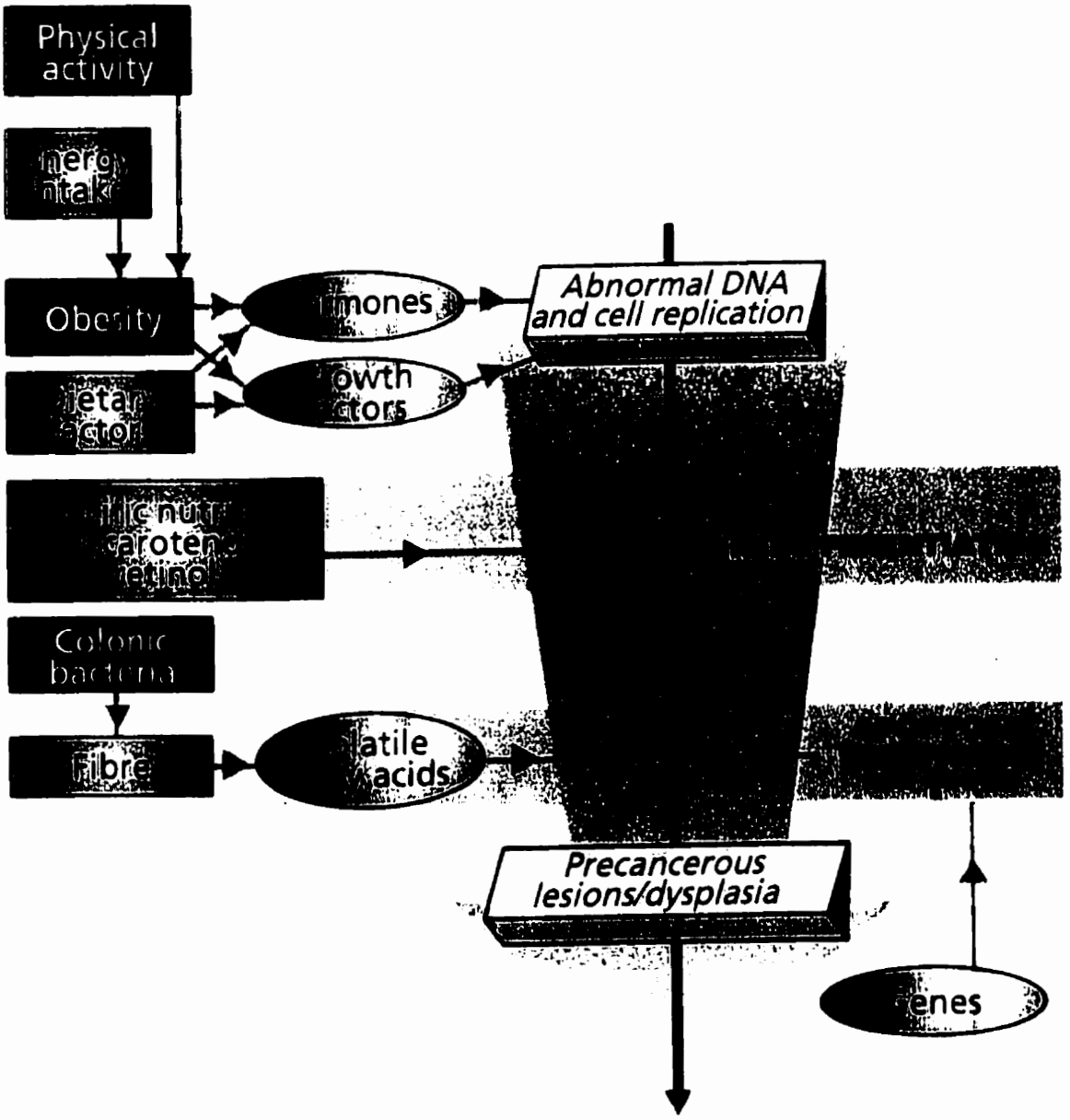
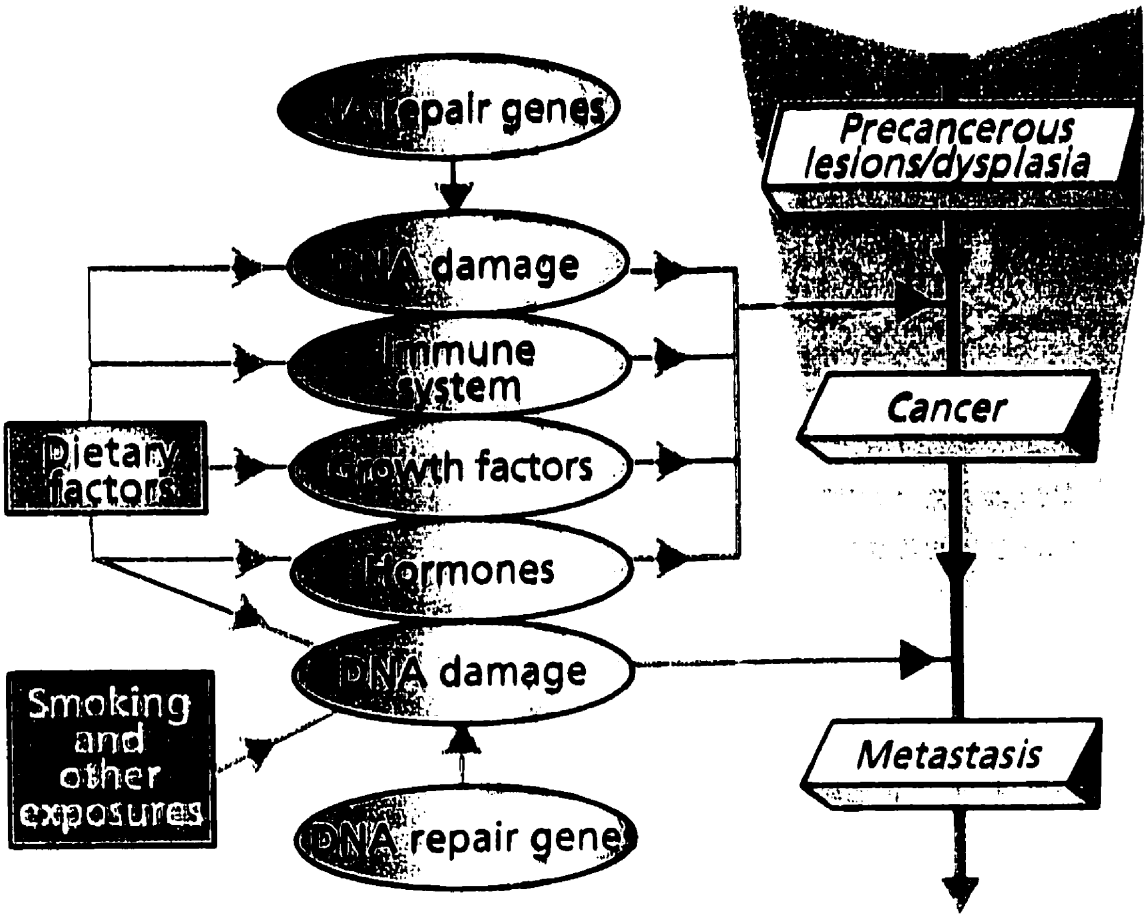

Physical activity
Obesity
Abnormal DNA and cell replication
Colonic bacteria
Fibre
Precancerous lesions/dysplasia

Figure 2.14 Influence of dietary factors on cellular endocrine and nuclear systems in the progression of precancerous lesions to cancer. Adapted from World Cancer Research Fund, 1997.


repair genes
damage
Immune system
Dietary factors
Growth factors
Hormones
DNA damage
Smoking and other exposures
DNA repair gene
Precancerous lesions/dysplasia
Cancer
Metastasis

mentioned previously, corn oil is rich in linoleic acid that forms AA, the main precursor for eicosanoid synthesis. Prostaglandins are known to be involved in the induction of tissue ODC activity, and compounds that block prostaglandin formation, also block ODC induction (Verma et al., 1980). Such studies provide the impetus toward exploring the effect of dietary lipids on other critical signaling proteins involved in colon carcinogenesis.

### 2.5 Summary

The pathological evolution leading to cancer of the colon is under the influence of many genetic and environmental factors. The discovery and application of the ACF system allows for assessment of suspected dietary modulators on the multiple precancerous stages in the colon, at a single time point. Epidemiological evidence indicates that a diet high in fat may be positively associated with colon cancer incidence. Although numerous studies have investigated the effect of dietary lipids on end-stage tumor incidence in experimental animal models, there is limited information regarding the growth mechanisms leading to these findings. In addition, variations in experimental design regarding the timing of diet and carcinogen administration create difficulties when comparing studies. Simultaneous intervention of ACF at different developmental stages with diets of varying fatty acid composition will provide insight as to whether specific stages of colon cancer are more susceptible to dietary modulation.

Mounting biochemical evidence indicates that dietary lipids modulate many critical signaling enzymes involved in the carcinogenic process. However, many gaps exist regarding the effect of lipids on proteins responsible for the initiation of

signaling messages from the cell surface. Furthermore, how these cellular messages are interpreted at the nuclear level, potentially affecting gene expression and translation, is not known. It is well established that complex interactions occur between signaling elements. The inhibition of one pathway may lead to increased activation of another in order to compensate, and vice-versa. This complexity is further intertwined with the uncontrolled cellular events implicated in cancer development.

The scientific approach undertaken in this dissertation has stemmed from the integration of many lines of evidence detailing our existing knowledge of normal and abnormal cellular growth. Each study was designed in attempt to further refine our understanding of the role of dietary lipids in the etiology and pathogenesis of colon cancer.

# CHAPTER 3

# MATERIALS AND METHODS

The materials and methods described are common to several studies in this dissertation. Most chemicals were purchased from Sigma Chemical Co, Mississauga, ON, Canada unless otherwise noted.

## *3.1 Colon Carcinogen*

The colon specific carcinogen azoxymethane (AOM) was dissolved in 0.9% saline and used in all experiments. Animals were injected s.c. once weekly for three weeks at a concentration of 15 mg/kg BW.

## *3.2 Quantification of ACF*

All rats were killed by $CO_2$ asphyxiation. Colons were removed, flushed with cold phosphate-buffered saline (PBS), slit from cecum to anus and fixed flat in filter paper in 70% ethanol at 4°C. All filter papers were coded in order for colons to be scored blindly. After a minimum of 24 hours, colons were scored for ACF by staining in 0.2% methylene blue in PBS for 5 minutes. ACF were quantified by light microscopy using the method first described by Bird (1987). The whole mount of colon was placed on a glass slide and viewed mucosal side up under 10X magnification. ACF were distinguished from normal crypts by their increased size, elongated luminal opening, increased thickness of the epithelial lining and increased pericryptal zone (Bird, 1995).

The number, distribution and multiplicity of ACF were determined along the entire colon length. To determine distribution, the number of ACF in every 2 cm

section of the colon starting at the rectal end was recorded. To determine crypt multiplicity, the number of crypts in each foci was recorded and analyzed in two ways. Average crypt multiplicity representing the average number of crypts in a focus per colon or per group was determined. ACF with different growth features were categorized based on their crypt multiplicity as having small (1-3 crypts/focus), medium (4-6 crypts/focus) or large ACF (≥ 7 crypts/focus).

### *3.3 Tumor Assessment*

The location, appearance and dimensions of all suspicious lesions were recorded. Suspected tumors and microadenomas were dissected out with 0.5 cm surrounding mucosa, and fixed in 4% paraformaldehyde or 70% ethanol for immunohistochemical purposes. Alternatively, tumors were dissected out without attached normal mucosa and snap-frozen in liquid nitrogen for the isolation of RNA and protein.

Tumor assessment parameters have been described previously (Bird et al., 1996). Briefly, they include tumor incidence (percentage of total animals with tumors); tumor multiplicity (average number of tumors per tumor-bearing rat); total tumors per group; average tumor size ($mm^2$) per tumor-bearing rat; average tumor size/group; and tumor burden (average of total tumor area in each tumor-bearing rat). Sample calculations are provided below:

1) Average tumor size per tumor-bearing rat:

$$\frac{\text{Total of average area } (mm^2) \text{ of tumor in each tumor-bearing rat in each group}}{\text{Number of tumor-bearing rats in that group}}$$

2) Average tumor size/group:

$$\frac{\text{Total tumor size } (mm^2) \text{ in a group}}{\text{Number of tumors in that group}}$$

3) Tumor burden:

$$\frac{\text{Total tumor area in each tumor-bearing rat in group}}{\text{Total tumor number in that group}}$$

### *3.4 Cell Proliferation*

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 colons fixed in 70% ethanol were used. The tissues were embedded in paraffin wax on edge and sectioned at a thickness of 5 μm. Longitudinal unstained colonic crypt sections were mounted onto slides. Immunohistochemical techniques were carried out using the unlabelled antibody bridge method and the bulk Histo-Stain SP kit from Zymed (London, ON, Canada). Tissue sections were deparaffinized in xylene, rehydrated and flooded with normal goat serum and incubated for 20 minutes to block non-specific binding. Anti-PCNA monoclonal antibody (Dimension Laboratories Inc, Mississauga, ON, Canada) was diluted in PBS (1:40), applied to the sections and incubated for 1 hour. The sections were then incubated with anti-mouse IgG (antibody bridge) for 20 minutes, followed by incubation with mouse IgG peroxidase (labeling agent) for 20 minutes. The peroxidase reaction was initiated by immersing the slides in 0.06% 3,3-diaminobenzidine tetrahydrochloride with 0.03% $H_2O_2$ added for 5-10 minutes. The slides were rinsed 3 times with fresh $H_2O$ and counter stained with

haemotoxylin, sequentially dehydrated and mounted with Permount (Fisher Scientific, Ottawa, ON, Canada). Incubations throughout the procedure were carried out in a humid, ambient chamber at room temperature, and slides were washed extensively with fresh PBS between incubations. PCNA-labeled cells were determined in ten crypts per colon and classified as darkly stained cells along the length of the entire crypt. The number and position of the labeled cells in each crypt were recorded. All slides were coded to ensure they were scored blindly. The proliferative zone was defined as the highest labeled 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. Crypt height (CH) was defined as the number of cells per mid-axial crypt. PCNA labeling index (LI) was calculated as the number of labeled cells per crypt divided by the total number of cells along both sides of the crypt and multiplied by 100.

### *3.5 Protein Analysis*

Protein concentration was assessed according to the Bradford method (1976) using a Coomassie protein assay reagent (Pierce, Rockford, IL, USA). Bovine serum albumin was used as standard. Standard and experimental samples were analyzed in duplicate using a Spectra Max 3000 (Molecular Devices, Sunnyvale, CA, USA).

### *3.6 Detection of Antigens by Western Blotting*

Mucosal cell lysates were extracted and protein concentration determined as stated previously. The buffer systems and gel recipes for western blotting techniques are described in Appendix E. Detection of TGF-$\alpha$ will be used as an

example. An equal amount of protein for each sample was combined with an equal volume of 2X sodium dodecyl sulphate (SDS) sample buffer and boiled for 5 minutes at 95°C. Samples were separated on a discontinuous denaturing gel system using 0.75 mm 5% stacking and 15% separating SDS-PAGE (polyacrylamide) gels as per the method of Laemmli (1970). Electrophoresis was run at 170 volts for 70 minutes using a molecular weight standard (Gibco BRL) to track sample separation. The transfer of proteins from gel to membrane was performed in a 20% methanol buffer at 120volts for 150 minutes at 4°C using a BIO-RAD Miniprotean cell (Mississauga, ON, Canada). The 0.45-micron nitrocellulose hybond-C membrane (Amersham Life Sciences, Arlington Heights, IL, USA) was soaked for 1 hour in distilled $H_2O$, and in transfer buffer for 10 minutes prior to transfer. To ensure equal loads and even transfer of proteins, the gels were stained with Coomassie blue. The membrane was blocked, with rocking, with 5% skim milk powder (SMP) in TBS-T (Tris buffered saline with 0.1% Tween-20) for 1 hour at room temperature. The membranes were then washed for 40 minutes with at least 3 changes of TBS-T (50 ml). Membranes were then probed with primary antibody (polyclonal TGF-α, cat# sc-36, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000 in 5% SMP+TBS-T overnight at 4°C. After incubation with the primary antibody, the membrane was washed as described previously. Incubation with the secondary antibody, mouse anti-goat horseradish peroxidase (HRP) conjugated IgG was performed at a dilution of 1:1000 in 5% SMP+TBS-T for 1 hour at room temperature. The membrane was then subjected to a final wash with TBS-T. Immunoreactivity was

detected using an enhanced chemiluminescence kit (ECL, Amersham). Film (Kodak x-OMATAR) was exposed to the illuminating membrane for 5 minutes. Densitometric analysis was performed with ScionImage 2.0 software for microcomputers. To ensure the detection of immune-complexes by ECL were linear, initial trials were conducted utilizing increasing amounts of protein for each antibody (5-100 μg). The amount of protein loaded for each antibody was determined based on the median of the linear range. An equal number of samples per group were loaded per gel to control for any gel-to-gel variation. This allowed for comparison of expression from one film to another for the same antibody.

### *3.7 Preparation of Samples for EGFR-TK Activity*

The initial method for the preparation of colonic mucosal scrapings for the assessment of EGFR-TK activity was obtained directly from the lab of Dr. A. Majumdar (Wayne State University, Detroit, MI, USA). Mucosal scrapings were removed from storage at -80°C and thawed on ice. 0.3 grams of tissue was taken from all samples and placed in 3 ml ice-cold cytosolic buffer containing 25 mM Tris, O.25 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EGTA, 15 mM mercaptoethanol, 0.25 M sucrose, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 40 μg/ml aprotinin and 10 μg/ml trypsin inhibitor. All tissues were then homogenized with a polytron and centrifuged at 33,000 RPM (100,000 x *g*) for 1 hour. The supernatant was drawn as the cytosolic fraction and placed on ice. The membrane pellet was then resuspended in 3 ml of resuspension buffer containing 10 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF, 1 mM

$Na_3VO_4$, 1 mM 1,10-phenanthroline, 10 μg/ml leupeptin, 1 μl/ml aprotinin, 0.1% Triton-X and 0.5% Nonident P-40. A small aliquot was taken for protein analysis, and the remaining membrane fractions were immediately frozen at -80°C.

### *3.8 Immunoprecipitation of EGFR*

Immunoprecipitation was carried out according to the method of Malecka-Panas et al. (1996). Immunoprecipitation of EGFR from the membranous fraction was required in order to assess the specific tyrosine kinase activity associated with this receptor, as there are numerous other tyrosine kinases within the membrane. Initially, Pansorbin cells (Calbiochem, La Jolla, CA, USA) were centrifuged at 3000 x *g* for 10 minutes, then suspended in an equal volume of PBS containing 10% w/w mercaptoethanol and 3% w/w SDS. The cells were then boiled for 30 minutes, and centrifuged for 10 minutes at 3000 x *g*. The cells were washed extensively with PBS to remove any remaining mercaptoethanol/SDS solution, and used for the immunoprecipitation procedure.

In order to assess stimulated EGFR-TK activity, 200 μg of protein was taken for each sample and incubated with 6 μM ATP and 1 x $10^{-8}$ M of TGF-α for 15 minutes on ice. Another 200 μg of protein was taken for each sample, and not incubated with TGF-α to represent basal levels of EGFR-TK activity. The reaction was terminated by the addition of an equal volume of RIPA buffer (see Appendix D) with 1 μl of polyclonal anti-EGFR sheep antibody (UBI, Lake Placid, NY, USA) to all samples. This antibody was selected, as it does not bind to the tyrosine kinase domain of the receptor. All samples were incubated for 2 hours, with rocking, at 4°C with the antibody.

After 2 hours, 35 µl of treated pansorbin cells were added to each sample and incubated for 2 hours, with rocking, at 4°C. The membrane fractions were then centrifuged for 10 minutes at 10,000 x *g*. The supernatant was removed, and the remaining pellets were resuspended in 60 µl of resuspension buffer and split into two 30 µl samples (in order to have a duplicate reading) and kept on ice until the time of the assay.

### *3.9 EGFR-TK Activity*

Assessment of EGFR-TK activity was performed immediately following immunoprecipitation. Each 30 µl sample was incubated with 20 µl of reaction cocktail containing 2.5 µl IBS (1 M Tris pH 7.8, 1 M $MgCl_2$, 2mM $Na_3VO_4$), 3 µl ATP (60 µM) and 12.5 µl of a glutamic acid: tyrosine polymer (4:1, 12.5 µg/5 µl), and 2 µl $\gamma^{32}$ ATP (0.5 µCi, 3000 Ci/mmol). Incubation was conducted for 10 minutes at room temperature. The reaction was then stopped by spotting the entire reaction volume (50 µl) onto filter paper (P81, Whatman) and placed immediately in scintillation vials containing 20 ml of 10% trichloroacetic acid (TCA) with 0.2% sodium pyrophosphate (NaPP). Samples were washed once and then incubated overnight at 4°C in 20 ml of TCA+NaPP solution. The following morning, all samples were washed three times with TCA+NaPP, placed in 5 ml of Cyto-Scint scintillation fluid (Fischer Scientific), and read for one minute of a Beckman L-6000 Scintillation Counter. The blank was prepared by conducting the assay of 35 µl of pansorbin cells in resuspension buffer, and was done in duplicate. Results were calculated as the average between duplicates minus the blank, and expressed as pmol Pi transferred/100 µg protein.

### *3.10 Statistical Analyses*

All statistical analyses was carried out using the Statistical Analysis System (SAS) for microcomputers, version 6.06 (SAS Institute Inc., Cary, NC, USA) and are described for each experiment. A level of $P \leq 0.05$ was considered significant, unless otherwise indicated.

# CHAPTER 4

# MODULATION OF COLONIC EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) ACTIVATION AND PROLIFERATIVE STATUS BY DIETARY LIPIDS: A PRELIMINARY INVESTIGATION

## 4.1 Introduction

The human gastrointestinal tract is one of the most highly proliferative organs, as the entire epithelial lining is renewed every 4-8 days. The balance of cell growth and differentiation in the large intestine is maintained by numerous regulatory compounds, including growth factors. The epidermal growth factor (EGF) family of growth factors have been implicated in the control of cell proliferation in the colonic epithelium. The EGF family of peptides consists of several members that share both sequence homology and affinity for their receptor EGFR. One peptide in particular, transforming growth factor-alpha (TGF-α), is highly expressed throughout the colonic mucosa at all stages of development (Hormi & Lehy, 1994; Perez-Thomas et al., 1993). TGF-α is proposed to be involved in cell proliferation by binding with EGFR, activating its intrinsic tyrosine kinase activity, leading to the activation of other signaling intermediates to the nucleus. TGF-α has also been demonstrated to be the most potent activator of EGFR-TK in normal rat colonic mucosa compared to other EGF family ligands (Malecka-Panas et al., 1996).

The significance and measurement of cell proliferation in the colon has been an ongoing investigation, as it is believed that an increase in proliferation is

an indication of increased cancer risk. Various techniques are available to assess the proliferative status of the colonic mucosa, including the quantification of proliferating cell nuclear antigen (PCNA) as an indicator of cycling cells. Previous studies have demonstrated that composition of the experimental diet can affect cell proliferation in the rat colon. A diet high in fat has been suggested to increase mucosal cell proliferation compared to a low fat diet (Bird & Stamp, 1986). However, inconsistent results have been found among studies (Bird & Lafave, 1995; Robblee & Bird, 1994). Clinical studies have shown that fish oil supplementation decreases colonic mucosal proliferation in healthy individuals (Bartram et al., 1993). It is thought that the potential protective characteristics of fish oil against colon cancer may be due to its anti-proliferative effects.

It is evident that dietary lipids alter mucosal cell proliferation, and that activation of EGFR by TGF-$\alpha$ is involved in initiation of epithelial cell cycling. However, it remains unclear as to whether dietary lipids can affect the production of TGF-$\alpha$, and the interaction of EGFR and its ligand at the cell surface. Plasma membrane fatty acid composition has been shown to reflect the composition of lipid in the diet (LaFave et al., 1994). Diets high in beef tallow and corn oil have been previously demonstrated to affect the activity of protein kinase C (PKC) in rat colonic epithelium (LaFave et al., 1994). Therefore, it is possible that changes in membrane structure may influence the activation of specific membrane-bound receptors such as EGFR.

This preliminary study was conducted to gain evidence in support of the concept that dietary lipids that are capable of altering PKC activity, would also

affect EGFR-TK activity, TGF-α expression, and colonic cell proliferation. The main objectives of this preliminary study were as follows: a) to determine if fatty acid composition of the diet has any influence on the activation of EGFR by TGF-α ; b) to assess the amount of TGF-α present in normal colonic mucosa; c) to quantify the proliferative status of the colon as affected by dietary lipids.

## 4.2 Materials and Methods

*Animal Care and Experimental Diets*

Weanling male Fischer 344 rats were purchased from the Central Animal Care Facility (University of Manitoba). Animals were housed in wire mesh stainless steel cages (3 rats/cage) with sawdust bedding using a 12:12-hour light-dark cycle. Temperature and humidity were kept constant at 22°C and 55% respectively. Animals had free access to food and water at all times, and were cared for in accordance to the guidelines of the Canadian Council of Animal Care. All experimental diets were based on the semi-synthetic AIN-76A diet (American Institute of Nutrition, 1977), as described in Table 4.1 and Appendix B. For the high fat diets, an additional 15% fat by weight was added as corn oil (HFC, Mazola Brand, Canada Safeway), beef tallow (HFB, Maple Leaf Foods Inc, Winnipeg, MB, Canada) or menhaden oil (HFF, Zapata Protein Inc, USA) at the expense of an isocaloric amount of carbohydrate. The AIN-76A diet containing 5% corn oil was used as control (LFC). The fatty acid composition of the dietary lipids and experimental diets is described in Appendix C.

**Table 4.1. Composition of Experimental Diets[1]**

| Component | LFC[2] | HFC | HFF | HFB |
|---|---|---|---|---|
| Casein | 20.0 | 23.0 | 23.0 | 23.0 |
| Corn Starch | 52.0 | 33.8 | 33.8 | 33.8 |
| Dextrose | 13.0 | 8.5 | 8.5 | 8.5 |
| Corn oil | 5.0 | 23.0 | 5.0 | 5.0 |
| Test oil | 0.0 | 0.0 | 18.0 | 18.0 |
| Cellufil | 5.0 | 5.9 | 5.9 | 5.9 |
| DL-Methionine | 0.3 | 0.3 | 0.3 | 0.3 |
| Choline bitartrate | 0.2 | 0.2 | 0.2 | 0.2 |
| AIN-76A mineral mix | 3.5 | 4.1 | 4.1 | 4.1 |
| AIN-76A vitamin mix | 1.0 | 1.2 | 1.2 | 1.2 |

1. Values represent the percentage composition
2. Abbreviations are as follows: LFC, low fat corn oil; HFB, high fat beef tallow; HFC, high fat corn oil; HFF, high fat fish oil.

*Experimental Design*

Animals were acclimatized for one week and given rat chow prior to the initiation of the experiment. Rats were randomly allocated to four experimental diets described previously (10 rats/group). Initial and weekly body weights were recorded. All animals were fed the diets for 6 weeks, and were then terminated by $CO_2$ asphyxiation. Colons were removed, flushed with cold PBS, slit from cecum to anus and placed on a cooled metal surface. 1 $cm^2$ sections were taken from mid and rectal regions of the colon and fixed in 70% ethanol. Mucosal scrapings were taken from the remaining colon, placed in sterile cryovials, immediately frozen in liquid nitrogen and stored at -80°C for approximately one month.

*Extraction and Separation of Colonic Cytosol and Membrane Fractions*

Colonic cell fractions were separated and prepared according to the methods described in chapter 3.

*Assay of EGFR-Tyrosine Kinase Activity*

Stimulation of EGFR by TGF-α, immunoprecipitation of the receptor, and quantification of EGFR-TK activity was performed as described in Chapter 3. Activity was expressed as pmol Pi transferred/100 µg protein.

*Detection of TGF-α Immunoreactivity by Western Blotting*

The separation and transfer of proteins was performed as described in Chapter 3. All samples were run on a single gel to maintain consistency. Recipes for polyacrylamide gels and buffers used for immunoblotting are

described in Appendix D. Area of the density of all immunoreactive bands was assessed and expressed as pixels.

*Assessment of Proliferative Status*

Proliferative status of the colonic mucosa was assessed by the detection of PCNA by immunohistochemistry. Calculation of the parameters used (crypt height, CH; labeled cells, labeling index, LI) is described in Chapter 3.

*Statistical Analysis*

Statistical analysis of the data was accomplished using SAS statistical software for microcomputers as described in Chapter 3. Differences among the groups were determined by analysis of variance in combination with Duncan's Multiple Range Test at $P \leq 0.05$.

## 4.3 Results

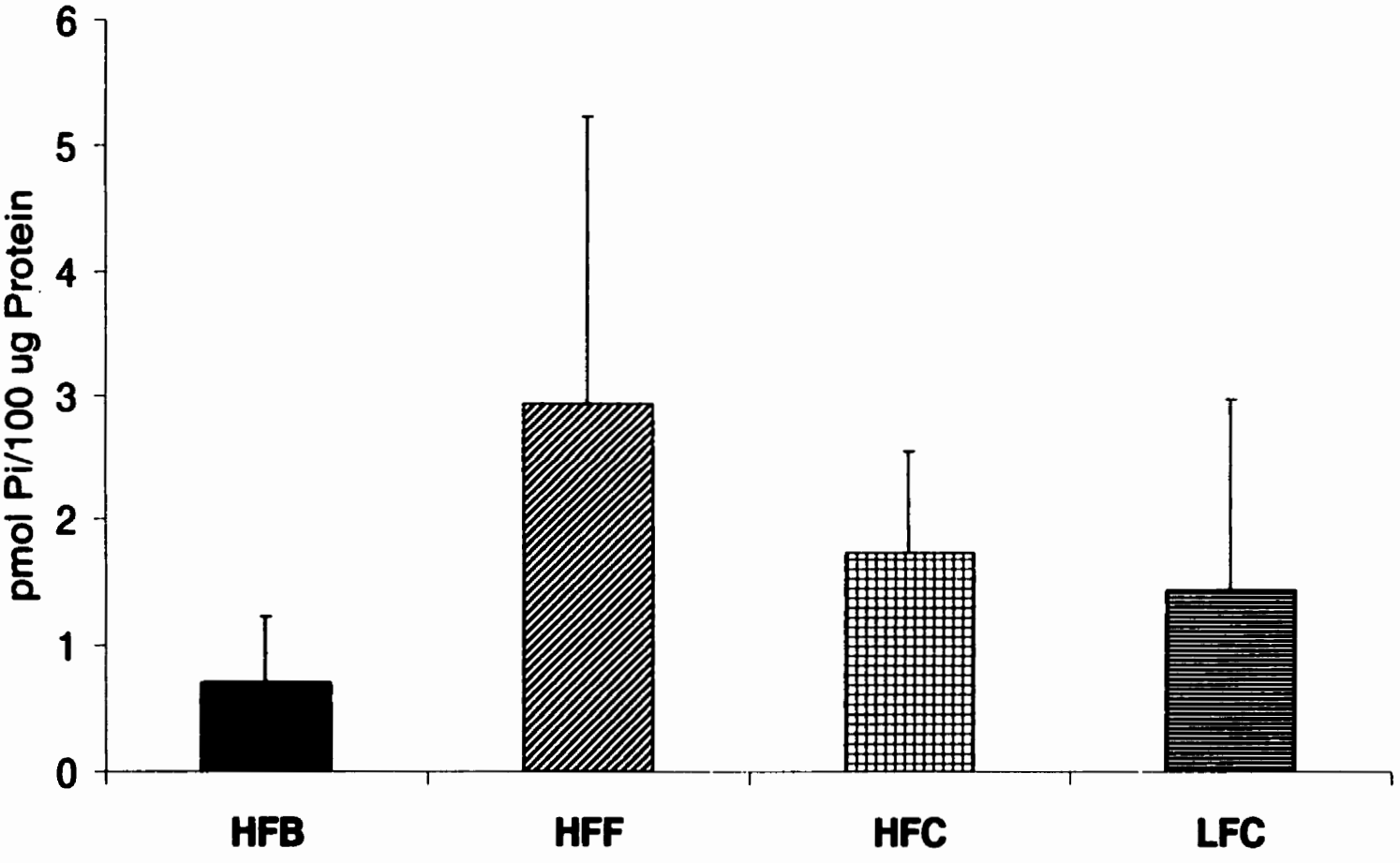
*Body Weights*

As expected, the animals fed the high-fat diets weighed more than those fed the low-fat control diet. However, there was no difference in weight among the high fat groups (data not shown).

*Colonic EGFR-TK Activity*

The HFF diet had the highest EGFR-TK activity of the high-fat diet groups, and was over 4-fold higher than the HFB group (Figure 4.1). The LFC control group was lower than the high fat groups, with the exception of HFB. There were no significant differences.

Figure 4.1 Quantification of TGF-α stimulated EGFR-TK activity in colonic mucosa from F344 rats fed HFB, HFF, LFC or HFC diets for 6 weeks (n=5 samples/group). Values are mean ± SD (bars) and are expressed in pmol Pi/100 μg protein.


pmol Pi/100 ug Protein
6
5
4
3
2
1
0
HFB
HFF
HFC
LFC

*Proliferative Status*

PCNA staining of the mid-section of the colon revealed that the LFC group had the highest number of labeled cells compared to the other high-fat diets, with HFC having the lowest number of labeled cells (Table 4.2). The average number of cells per crypt (expressed as crypt height) was highest, although not significantly, in the HFB group, followed by HFF, LFC and HFC. The overall PCNA labeling index (LI) was in the order of LFC>HFB>HFF>HFC.

The number of PCNA labeled cells in the rectal portion of the colon was highest in the HFF group of all the diet groups, as described in Table 4.2. There was no difference among the HFB, HFC and LFC groups. In terms of crypt height however, the HFC group was significantly lower than the remaining diet groups. The LFC and HFB groups had the equivalent value for CH, with HFF being only slightly higher. The overall labeling index for the rectal mucosa was in the decreasing order of HFF>HFC>LFC>HFB. There was no significant difference among the groups.

*Immunodetection of TGF-α*

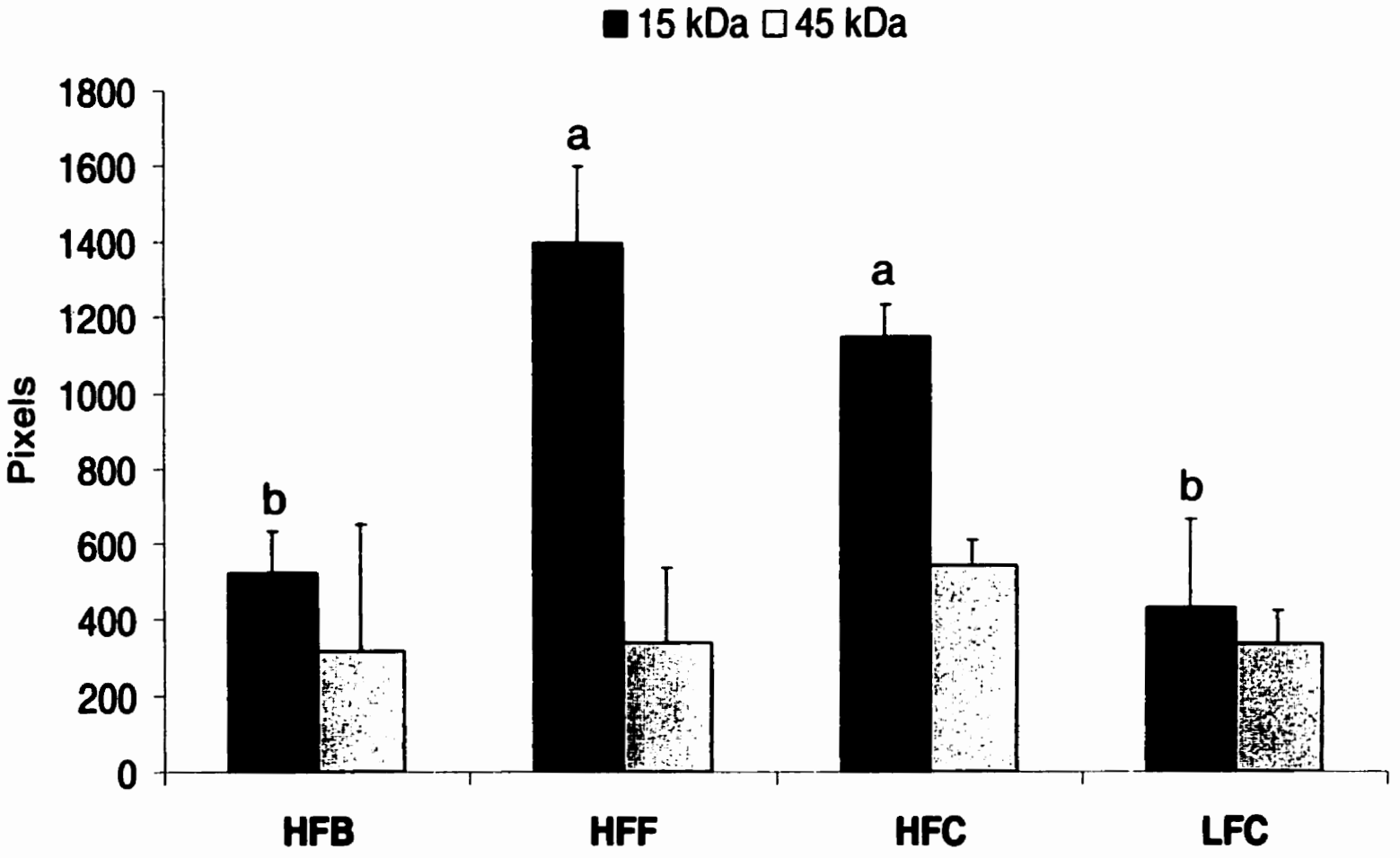
Immunodetection of TGF-α by western blotting revealed two distinct protein bands at 15 kDa and 45 kDa in all samples. Detection of the mature form of TGF-α (5 kDa) was absent in all samples. The average band density at 45 kDa was highest in the HFC group (539.33±70.1 pixels) with the density for the remaining groups being relatively equivalent (Figure 4.2). Analyses of the 15 kDa isoform of TGF-α demonstrated that the band density for the HFC and HFF groups, respectively, was significantly higher compared to the HFB and LFC

**Table 4.2 Proliferative status of normal colonic mucosa as affected by dietary lipids[1,2,3]**

| | HFF | | HFB | | HFC | | LFC | |
|---|---|---|---|---|---|---|---|---|
| | Distal[4] | Proximal | Distal | Proximal | Distal | Proximal | Distal | Proximal |
| Labeled Cells | 17.0 ±4.6$^{a}$ | 13.0 ±4.5$^{bc}$ | 14.4 ±3.9$^{abc}$ | 13.7 ±5.3$^{bc}$ | 14.9 ±6.9$^{ab}$ | 12.2 ±4.0$^{c}$ | 14.6 ±7.1$^{abc}$ | 14.4 ±6.0$^{abc}$ |
| Crypt Height | 25.0 ±2.2$^{a}$ | 23.3 ±2.2$^{bc}$ | 23.6 ±2.0$^{ab}$ | 23.6 ±3.0$^{ab}$ | 22.0 ±3.1$^{c}$ | 22.9 ±3.2$^{bc}$ | 23.6 ±4.7$^{ab}$ | 23.0 ±3.2$^{bc}$ |
| Labeling Index | 33.3 ±9.4$^{a}$ | 28.0 ±10.2$^{ab}$ | 30.0 ±7.6$^{ab}$ | 28.8 ±9.9$^{ab}$ | 32.9 ±14.3$^{a}$ | 26.7 ±8.3$^{b}$ | 31.0 ±14.5$^{ab}$ | 30.1 ±11.1$^{ab}$ |

1. Values represent mean ±SD
2. Values in the same row not sharing a common superscript are significantly different ($P \leq 0.05$)
3. Animals were terminated at 6 weeks of feeding the experimental diets. Abbreviations are as follows: HFB, high fat beef tallow; HFC, high fat corn oil; HFF, high fat fish oil; LFC, low fat corn oil.
4. Distal and proximal categories refer to different regions of the colon

Figure 4.2 Density of TGF-α protein expression in colonic mucosa from weanling F344 rats fed HFB, HFF, LFC or HFC diets for 6 weeks (n=5 samples/group). Immunoreactive bands were observed at 45 kDa and 15 kDa. Values are mean ± SD (bars) and are expressed in pixels, representing the area of the visualized protein bands. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).


15 kDa 45 kDa
Pixels
1800
1600
1400
1200
1000
800
600
400
200
0
a
a
b
b
HFB
HFF
HFC
LFC

groups, as described in Figure 4.2. The overall band density at 15 kDa was higher than the 45 kDa band for all diet groups.

## 4.4 Discussion

The present investigation explored the effects of various dietary lipids (beef tallow, corn oil, fish oil) on the expression of TGF-α and its ability to activate EGFR. An additional objective was to assess the proliferative responses of both proximal and distal colonic epithelium to these specific lipids.

The presence of TGF-α and its receptor has been demonstrated in normal and cancerous colonic tissues and cell lines (Cartlidge & Elder, 1989; Coffey et al., 1990; Derynck, 1988; Hormi & Lehy, 1994). It is thought that TGF-α acts as a potent mitogen in the colon by binding EGFR, activating its tyrosine kinase. A comparative analysis of rat mucosal EGFR-TK activity when stimulated by its various ligands (EGF, TGF-α, HB-EGF) concluded that TGF-α is the most potent stimulator of EGFR (Malecka-Panas et al., 1996). This evidence suggests that TGF-α may also play a more prominent role in the mitogenic activation of other signaling proteins, oncogenes and nuclear transcription factors than its sister ligands.

The mature 5.6 kDa form of TGF-α is derived from a 160 kDa precursor, a glycosylated protein attached to the cell membrane (Teixido et al., 1987). However, various isoforms of TGF-α have been reported in normal and cancerous tissues, ranging from 68 kDa to 12 kDa (Banerjee et al., 1998). Many of these higher molecular weight forms have been tested for biological activity, and have been suggested to activate EGFR through a juxtacrine pathway (Ignotz

et al., 1986; Linsley et al., 1985). In the present study, we utilized an antibody known to react with both mature and precursor forms of TGF-α. Despite the lack of detectable mature TGF-α in the colonic mucosa, two quantifiable bands were observed at 15 kDa and 45 kDa. Although the presence of mature TGF-α has been detected in colonic cell lines, few studies have reported the presence of the 5.6 kDa form of TGF-α in cell lysates from whole colonic tissue. This could be due to the fact that low levels of the mature form exist in the colonic mucosa, or that low molecular weight TGF-α may be degraded during the processing of tissue samples. Studies examining TGF-α expression by immunoblotting in rat prostate and hepatic tissues have observed similar larger molecular weight forms of TGF-α, but could not detect the mature form of the growth factor (Banerjee et al. 1998; Tanno & Ogawa, 1994).

The present study was the first to examine the effects of dietary lipids on the presence of TGF-α in rat colonic mucosa. The finding that there was a significantly higher amount of TGF-α in the HFF and HFC groups compared to the HFB and LFC groups suggests that high amounts of ω-3 and ω-6 fatty acids may play a role in the production and processing of TGF-α. The cleavage of pro-TGF-α into mature TGF-α is achieved by an elastase-like protease, whose activity is thought to be regulated by PKC (Pandiella & Massague, 1991a). PKC translocates from the cytosol to the membrane upon activation. A previous study has shown a similar proportion of membranous PKC activity in colonic mucosa from rats fed diets high in fish oil and corn oil (Good et al., 1997). PKC is also known to be a part of cell signaling pathways leading to the nucleus,

affecting cellular gene expression. It is therefore possible that the increased amount of immunoreactive TGF-α in the HFF and HFC groups could be as result of an increased cellular capacity to express and process premature TGF-α. It should be noted that the HFC group did demonstrate a higher amount of the 45 kDa isoform of TGF-α compared to the other diet groups, although the differences were not significant. Whether the various isoforms of TGF-α possess different biological activity remains to be explored.

Cellular membrane phospholipid composition has been shown to reflect the fatty acid composition of the diet in both humans and experimental animals. Subsequently, alteration of the membrane lipid composition can affect the function of the plasma membrane, as well as the proteins embedded with it. The activity of certain membrane-associated proteins, such as the insulin receptor, has been shown to increase in proportion with an increase in dietary linoleic acid (Field et al., 1989). The present findings demonstrate that the dietary fatty composition does influence the activation of EGFR associated tyrosine kinase by TGF-α. EGFR-TK activity was highest in the HFF group, although not significantly different from the other dietary groups. It was interesting to note that the HFB group was much lower than the other dietary groups by at least 50%. Beef tallow is a highly saturated and more solid fat compared to other experimental lipids (corn oil and fish oil). A diet high in saturated fat may decrease the fluidity of the plasma membrane, and therefore the ability of membrane-bound receptors to achieve specific conformational changes

associated with ligand binding and kinase activation, or affects the initial ligand-receptor interactions.

The HFC and HFF groups demonstrated increased EGFR-TK compared to the LFC control group. These diets also had the highest total levels of immunoreactive TGF-α, which may result in a higher initial level of EGFR activation prior to incubation with TGF-α during the experimental assay. Previous studies have demonstrated no effect of linoleic acid on EGFR number and binding affinity in mammary epithelial cells (Bandyopadhyay et al., 1993). However, the fatty acid was merely added to the cell culture medium along with EGF to stimulate the receptor, and therefore does not directly represent the chronic membrane-associated affects of lipids on EGFR activation. Consequently, this study and the previous study are limited by the fact the actual number and binding-affinity of EGFR was not assessed, and also whether linoleic acid was incorporated into the plasma membrane.

The role of proliferation in malignant potential and the appropriate protocol used to assess proliferation has been a subject of debate for many years (Farber, 1995). In the present study, the method used to measure proliferative status of the colon was the expression of PCNA. This method is considered to be the preferred choice as it is noninvasive and does not require the use of cytotoxic chemicals (such as BrdU), and is therefore ideal when enzymatic and proliferative measurements are to be evaluated in the same colonic sample. Comparative studies have demonstrated that a strong correlation exists between BrdU and PCNA is the assessment of proliferative status in the colon. The

number of PCNA labeled cells was higher in the distal portion of the colon for all groups compared to the proximal region. This trend was also seen in terms of crypt height (with the exception of HFC) and the overall LI (with the exception of HFB). It has been repeatedly demonstrated that the regions of the colon are distinct in terms of crypt architecture, sensitivity to carcinogen treatment, and biological responses to chemopreventive agents (Bird et al., 1996; Liu et al., 1995). In both humans and animal models, the highest colonic tumor incidence is often found in the distal colon. Therefore, these findings support that contention, as the rectal portion of the colon had a higher overall proliferative status.

The ability of fish oil to decrease colonic proliferation has been reported in numerous studies, and has been suggested as the underlying mechanism of its non-tumor promoting characteristics. From this perspective, it is interesting that animals fed the HFF diet had the highest distal proliferative status in all categories compared to the other diet groups. Furthermore, the diet-related changes in the distal colon LI followed the same trend as the EGFR-TK activity (HFF to HFB), suggesting that mucosal proliferation is positively associated with EGFR stimulation. Therefore, there appears to be an association between PCNA-LI and EGFR-TK activity. It should be noted that a similar positive association between EGFR-TK and colonic proliferative status was reported in rats following AOM exposure (Malecka-Panas et al., 1995). In summary, this preliminary study demonstrates that EGFR-TK activity could be involved in the diet-associated changes in colonic crypt proliferation indices.

# CHAPTER 5

# AN INVESTIGATION INTO THE GROWTH REGULATORY EFFECT OF DIETARY LIPIDS ON PRECANCEROUS LESIONS REPRESENTING DIFFERENT STAGES OF CARICINOGENESIS IN RAT COLON

## 5.1 Introduction

The role of diet in the etiology and prevention of colon cancer has been substantiated by both epidemiological evidence, and studies in animal models (Howe et al., 1997; Reddy, 1994; Slattery et al., 1997). Several studies have demonstrated that diets high in corn oil, safflower oil, and beef tallow promote the development of tumors in rodents treated with a colon-specific carcinogen (Bird et al., 1996; Bird & LaFave, 1995; Reddy, 1994). However, when the source of dietary fat was derived from fish oil or coconut oil, the tumor-promoting effects were not seen (Bird & LaFave, 1995; Reddy, 1994). These data suggest that the ability of specific dietary lipids to enhance colonic tumor development is linked to their characteristic fatty acid composition.

It is well recognized that cancer development is a multistep process involving a continuum of preneoplastic stages that are microscopic and macroscopic in dimension. To prevent the appearance and progression of cancer, it is pivotal to understand the amenability of these precancerous stages to modulation by dietary, and potentially preventive, agents. Aberrant crypt foci (ACF) are present in carcinogen-treated rat colons and are thought to represent preneoplastic colonic lesions (Bird, 1995). Several reports have supported the

preneoplastic nature of ACF, and use of the ACF system to quantify the stepwise development of colon cancer and identify modulators of colon carcinogenesis (Bird, 1995; Bird et al., 1996; Lasko et al., 1999; Wargovich et al., 1996).

In a majority of chemopreventive studies exploring the role of nutrients on colon cancer development, animals are exposed to a cancer-modulating nutrient or test agent during or soon after carcinogen treatment. This exposure is continued throughout the study duration, ranging from a few weeks to several months. Upon completion of the study, final tumor incidence and the number of ACF are assessed. In this approach, newly initiated preneoplastic lesions are exposed to the experimental diet, which may interact or confound the effects of the newly administered carcinogen (Wargovitch et al., 1996). Therefore, one can suggest that the altered tumor outcome resulting from treatment with an experimental diet or nutrient before, during or soon after carcinogen injection is due to modulation of the early events of colon carcinogenesis. These events may include alteration in the growth of preneoplastic lesions while they are in the early stages of development, or stimulation of a selected population of initiated cells, thus selectively inhibiting or promoting their clonal expansion.

Many studies have established the biological complexity of preneoplastic colonic lesions (Bird, 1995; Lasko & Bird, 1995; McLellan & Bird, 1988; Pretlow et al., 1992; Wargovitch et al., 1996). It has been demonstrated that advanced ACF display increased resistance to apoptotic cell death and the cytotoxic effects of bile acids compared to their less advanced counter parts (Bird, 1995b;

Magnuson et al., 1994). A recent study demonstrated that the growth-modulating ability of a low-or high-fat diet is established during the early stages of colon carcinogenesis (Bird et al., 1996). Therefore, it is reasonable to suggest that, depending on their stage of development, precancerous lesions may respond differently to the growth-modulatory effects of specific nutrients, such as dietary lipids. Furthermore, it is logical to propose that an agent that prevents the development of tumors when administered during or soon after carcinogen treatment may do so by preferentially inhibiting the growth of primal preneoplastic lesions, and may not be effective in inhibiting the growth of advanced lesions.

This situation may occur in high-risk individuals for colon cancer who already harbor preneoplastic lesions of various dimensions, thereby reinforcing the importance of studying the growth responses of established lesions to dietary agents. The experimental approach taken in the current study using the ACF system was to mimic the pathological situation encountered in the colons of high-risk individuals. In this approach, rats are injected with a colon-specific carcinogen (AOM). Intervention with experimental diets is initiated 12 weeks after carcinogen treatment, and continued for an additional 12 weeks. The rational behind this approach is that at the time of dietary intervention, the colons of the experimental animals harbor a large number of ACF with varying crypt multiplicities. It is recognized that the crypt multiplicity (number of crypts per focus) of an ACF represents its developmental state.

The main objective of the present study was to investigate the amenability of preneoplastic lesions at different stages of development to the growth-

modulating effects of various dietary lipids. The dietary lipids used (beef tallow, corn oil, fish oil) were chosen due to their contrasting fatty acids composition and proposed effects on colonic tumor development. Growth features of ACF, microadenomas (MA) and tumor incidence were assessed before and after dietary intervention.

## 5.2 Materials and Methods

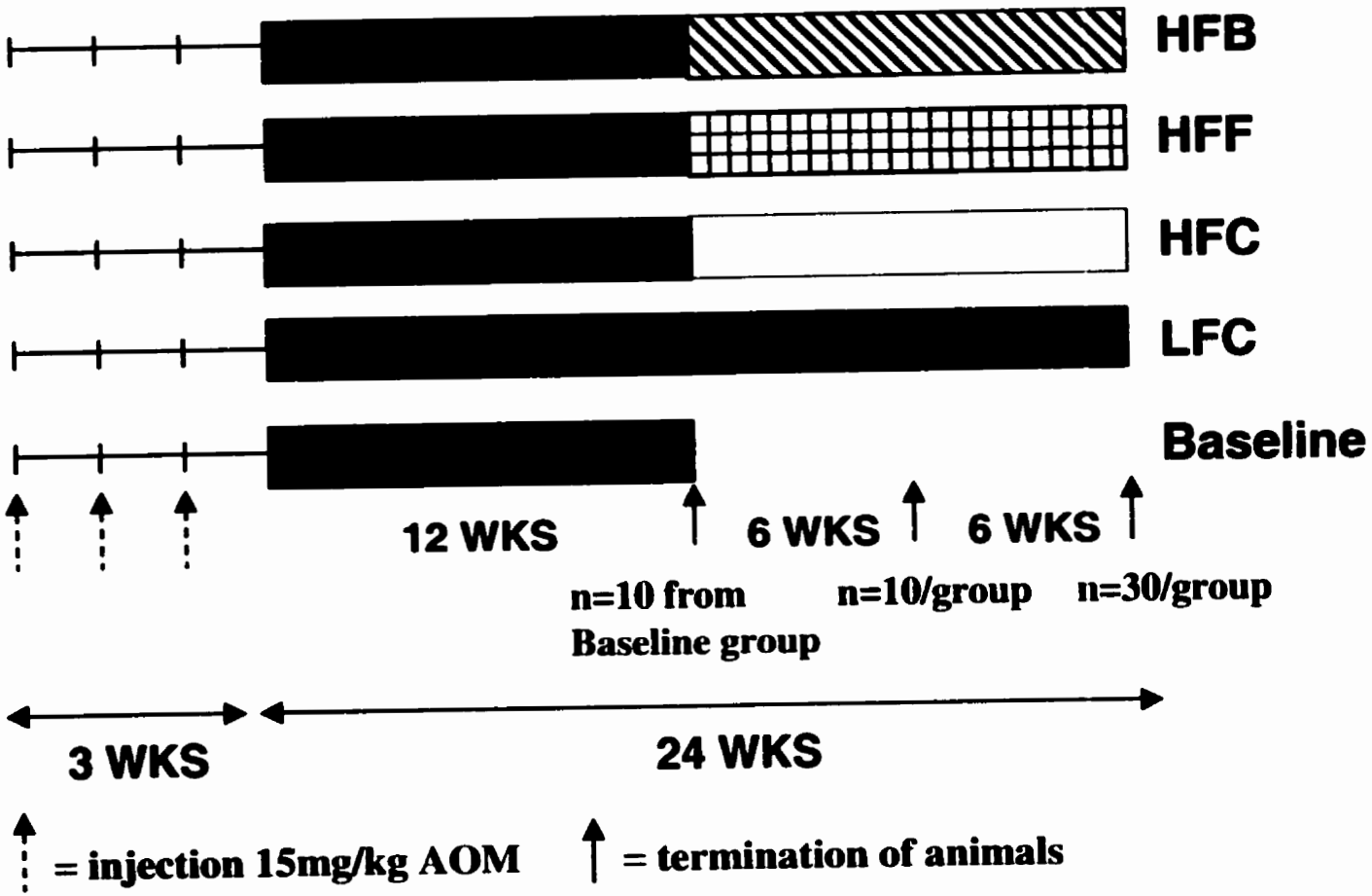
*Animals and Experimental Diets*

Male Fischer 344 rats were purchased from the Central Animal Care Facility (University of Manitoba) and acclimatized. All animals were approximately 90-100 g at the time of injection. The conditions of animal care were according to the Canadian Council of Animal Care as described in Chapter 4. Experimental diets were formulated on the basis of the AIN-76A diet (American Institute of Nutrition, 1977) and are described in Chapter 4. The descriptions of the diets and their fatty acid composition are supplied in Appendix C. The supply of beef tallow and corn oil, along with the prepared diets, were kept at 4°C. Menhaden oil was provided by Zapata Protein Inc, delivered weekly, and stored at -20°C until used. The prepared fish oil diet was tightly sealed and stored at -20°C to minimize oxidation. All diets were prepared twice weekly.

*Experimental Design*

A summary of the study protocol is detailed in Figure 5.1. Male weanling F344 rats were injected three times with AOM in fresh saline (15 mg/kg/wk). Animals were given free access to laboratory chow over the duration of the

Figure 5.1 Schematic representation of the experimental protocol (broken arrows designate injections with AOM, solid arrows indicate termination of animals). Animals were fed standard lab chow during carcinogen injection, and then given free access to control diet (LFC) for 12 weeks. At this time, 10 animals were killed to provide baseline data. The remaining animals were randomly divided to one of three diet groups HFC, HFB or HFF, while one group continued to receive the LFC diet. After 6 weeks of feeding (18 weeks after the last AOM injection), 10 animals per group were terminated. After 12 weeks of feeding (24 weeks after the last AOM injection), all remaining animals were terminated for all dietary groups (approximately 30 rats/group).


HFB
HFF
HFC
LFC
Baseline
12 WKS
6 WKS
6 WKS
n=10 from Baseline group
n=10/group
n=30/group
3 WKS
24 WKS
= injection 15mg/kg AOM
= termination of animals

injection period. One week after the third and final injection, all animals were placed on the low-fat corn oil diet (LFC, control). After 12 weeks of LFC feeding, 10 animals were killed by $CO_2$ asphyxiation, and their colons were assessed for ACF and adenomatous lesions. This was considered the baseline group and represented baseline values of ACF prior to diet intervention. The remaining animals were randomly subdivided into four diet groups. One group continued to receive the LFC diet, whereas the other three groups included an HFB, HFC and HFF diet. After six weeks of feeding the experimental diets, 10 animals per group were terminated, and their colons assessed for tumors and ACF. The remainder of the animals was terminated after week 12 of feeding (24 weeks after the last carcinogen injection). Tumor incidence was assessed for all animals in each group. Ten animals per group were designated for ACF analysis.

*Quantification of ACF*

Enumeration of the total number and crypt multiplicity of ACF per colon was performed as described in Chapter 3.

*Assessment of Tumors*

Tumor parameters were assessed according to the methods outlined in Chapter 3.

*Statistical Analysis*

Statistical analysis of the data was accomplished using SAS statistical software for microcomputers as described in Chapter 3. Differences among the groups were determined by analysis of variance in combination with Duncan's

Multiple Range Test at $P \leq 0.05$. Differences in tumor size and incidence among the experimental groups were determined by $\chi^2$ analysis.

## 5.3 Results

The body weights of the animals were affected by the different dietary lipids and were significantly different throughout the study; the means were in the order of HFF>HFC>HFB>LFC at the final termination (407.5g, 391.5g, 390.9g and 356.4g respectively, data not shown). The animals fed the high fat diets weighed more than those fed the LFC diet, although the differences were not significantly different. At the time when dietary intervention was initiated (after 12 weeks of feeding LFC diet), animals had an average of 350.1±61.1 ACF/colon. The crypt multiplicity of ACF ranged from 1 to 6 or more crypts/focus (Table 5.1). These results were used as baseline data prior to diet intervention and are represented as the Baseline group.

After 6 weeks of feeding the experimental diets, the colons of the animals were examined for preneoplastic changes and were compared with the changes that were present at the initial time of intervention. Size was used as the main criterion to distinguish MA from tumors. A lesion with several crypts that protruded from the surface and was $\leq 1mm^2$ was classified as MA. After 6 weeks of diet intervention, the HFF group had the highest average number of total ACF/colon, followed by HFC, HFB and LFC, although the differences were not found to be significant (Table 5.1). The HFB group had the highest average crypt multiplicity (CM) compared to the other diet groups. There was a trend for the

**Table 5.1 Enumeration of ACF Growth Characteristics in Male F344 Rats After 6 Weeks of Intervention with LFC, HFC, HFF and HFB Diets.[1,2,3]**

| Group | Total ACF[4] | CM | ACF 1-3 | ACF 4-6 | ACF ≥7 | MA |
|---|---|---|---|---|---|---|
| HFC | 507.6±120.9 | 2.4±0.3 | 422.1±103.8 | 72.11±23.2[b] | 13.3±8.9 | 13 |
| HFF | 586.9±140.5 | 2.5±0.2 | 473.1±108.6 | 104.3±25.6[a] | 16.4±10.6 | 16 |
| HFB | 488.5±129.4 | 2.6±0.4 | 392.1±118.7 | 95.4±28.9[ab] | 14.5±14.6 | 9 |
| LFC | 486.0±89.5 | 2.4±0.3 | 396.9±70.2 | 77.2±26.4[b] | 11.0±5.2 | 7 |
| Baseline | 350.1±61.0 | 2.9±0.1[a] | 216.9±49.7 | 77.4±19.1[b] | 10.3±5.1 | 0 |

1. Values are expressed as mean ± SD
2. Values in the same column not sharing a common superscript are significantly different ($P \leq 0.05$)
3. Animals were terminated after 6 weeks of diet intervention, or 18 weeks after the last injection of AOM
4. Abbreviations are as follows: ACF, aberrant crypt foci; CM, average crypt multiplicity/group; MA, microadenomas representing microscopic lesions ≤ 1mm$^2$; LFC, low fat corn oil; HFB, high fat beef tallow; HFC, high fat corn oil; HFF, high fat fish oil; Baseline, levels before diet intervention

HFF group to have the highest number of ACF of all growth classifications measured (ACF1-ACF≥7), and was significantly higher for ACF with 4–6 crypts/focus compared to the other diet groups with HFC being the lowest. The LFC and HFC groups behaved differently. In these groups, the increases were seen only in the total number of ACF, and ACF with 1-3 crypt multiplicity. Moderate increases were seen in the population of ACF≥7 among all groups. The total number of MA per group after 6 weeks of feeding was highest in the HFF group. The total number of MA per group in decreasing order was HFF (n=16) >HFC (n=13) >HFB (n=9) >LFC (n=7) as described in Table 5.1. Among the 10 rats examined in each group, there were 9-10 tumors per group (results not shown).

After 12 weeks of feeding the overall trend for the HFF group to exhibit the highest number of total ACF and ACF with advanced growth features persisted. A further increase in the total number of ACF was seen among all groups. The total average number of ACF/colon/group was in the order of HFF>LFC>HFB>HFC, and there were significant differences among the groups (Table 5.2). The HFB group continued to have the highest CM. The total average numbers of ACF with various crypt multiplicities representing different developmental stages continued to be the highest for the HFF group followed by HFB, LFC and the HFC group. The effect of HFF on the number of ACF with different growth features was significantly higher than the other groups with the exception of its effect on ACF 4-6 and ACF≥7 crypts/focus. Examination of the number of ACF with different growth features revealed that HFF, HFB and LFC

groups had increases in the number of ACF in all growth categories, whereas the HFC group had moderate increases in the number of ACF with 1-3, 4-6 crypt multiplicity only (Table 5.2). All animals were also examined for microscopic lesions. Although the HFF group had the highest number of total ACF and ACF with advanced growth features at both time points, this group had the lowest number of MA among the groups studied at the final termination point.

Tumor incidence was higher in the HFC and HFB groups compared to the LFC or HFF groups. The average size of tumors per tumor bearing rat or average tumor size per group was in the order of HFF>HFB>HFC>LFC (Table 5.3). Total tumor burden, which is the total tumor area per tumor bearing rat was also highest in the HFF group. Incidence of total macroscopic lesions, tumors and MA/rat was highest for the HFC group followed by HFB, HFF and LFC. Tumor multiplicity (representing the average number of tumors/tumor bearing rat) was in the order of HFB>HFC>LFC>HFF. The average tumor size/group was significantly higher ($P \leq 0.05$) in the HFF group compared to the other groups, with the HFC group being the lowest. The HFF group also had the highest average tumor size/tumor bearing rat and highest tumor burden (total tumor area/tumor bearing rat), although the levels were not significantly different.

All tumors were further categorized by size into small, medium and large tumors. The HFC and HFB groups had the highest number of small tumors (23 and 25, respectively), with the HFF group having the lowest number of small tumors (Table 5.4). The number of medium sized tumors, in decreasing order, was HFC>HFB>LFC>HFF. All tumors were identified as adenocarcinomas.

**Table 5.2 Enumeration of ACF Growth Characteristics in Male F344 Rats After 12 Weeks of Intervention with LFC, HFC, HFF and HFB Diets.[1,2,3]**

| Group | Total ACF | CM | ACF 1-3 | ACF 4-6 | ACF ≥7 | MA |
|---|---|---|---|---|---|---|
| HFC | 674.6±160.4$^{b}$ | 2.4±0.1$^{c}$ | 560.4±145.8$^{b}$ | 98.6±22.3$^{ab}$ | 16.1±7.6$^{ab}$ | 16 |
| HFF | 955.7±269.2$^{a}$ | 2.5±0.3$^{bc}$ | 769.3±221.7$^{a}$ | 156.0±47.5$^{a}$ | 30.1±22.2$^{a}$ | 10 |
| HFB | 750.1±173.7$^{ab}$ | 2.6±0.2$^{b}$ | 586.0±157.5$^{b}$ | 133.5±32.0$^{a}$ | 25.5±10.3$^{a}$ | 16 |
| LFC | 835.1±218.2$^{ab}$ | 2.4±0.1$^{c}$ | 690.3±194.3$^{ab}$ | 128.1±27.5$^{ab}$ | 22.0±5.8$^{ab}$ | 12 |
| Baseline | 350.1±61.0$^{c}$ | 2.9±0.1$^{a}$ | 216.9±49.7$^{c}$ | 77.4±19.1$^{b}$ | 10.3±5.1$^{b}$ | 0 |

1. Values are expressed as mean ± SD
2. Values in the same column not sharing a common superscript are significantly different ($P \leq 0.05$)
3. Animals were terminated after 12 weeks of diet intervention, or 24 weeks after the last injection of AOM

**Table 5.3 Tumor Parameters in Male F344 rats Fed HFC, HFB, HFF and LFC Diets[1,2,3]**

| | LFC[4] | HFC | HFF | HFB |
|---|---|---|---|---|
| Total number of Rats | 41 | 38 | 42 | 42 |
| Tumor Incidence | 48.7% | 65.7% | 47.6% | 57.1% |
| Tumor Multiplicity | 1.55±0.29 | 1.60 ±0.13 | 1.30 ±0.11 | 1.72 ±0.16 |
| Average tumor size ($mm^2$)/group | 13.9 ±0.03$^{ab}$ | 12.5 ±0.02$^{b}$ | 21.8 ±0.03$^{a}$ | 16.6 ±0.03$^{ab}$ |
| Average tumor size ($mm^2$)/tumor-bearing rat | 14.4 ±0.03 | 13.9 ±0.02 | 18.7 ±0.04 | 15.3 ±0.02 |
| Tumor burden | 20.7 ±0.05 | 20.8 ±0.04 | 27.4 ±0.06 | 23.5 ±0.03 |

1. Values are expressed as mean ± SD
2. Values in the same row not sharing a common superscript are significantly different ($P \leq 0.05$)
3. Animals were terminated after 12 weeks of diet intervention, or 24 weeks after the last injection of AOM

**Table 5.4 Classification of Tumor Size (%) in Male F344 Rats After 12 Weeks of Intervention with HFC, HFB, HFF and LFC diets[1,2]**

| | HFC | HFF | LFC | HFB |
|---|---|---|---|---|
| Small[3] | 56.0 (23) | 45.0 (13) | 61.5 (19) | 55.0 (25) |
| Medium | 29.0 (12) | 20.5 (6) | 22.5 (7) | 20.0 (9) |
| Large | 15.0 (6) | 34.5 (10) | 16.0 (5) | 24.4 (11) |
| Total Tumors[4] | 41 | 29 | 31 | 45 |

1. Values are expressed as: percentage of total tumors (actual number)
2. Animals were terminated after 12 weeks of diet intervention, or 24 weeks after the last injection of AOM
3. Categories are defined as follows: Small, $<10mm^2$; Medium, $<20mm^2$; Large, $>20mm^2$
4. Values represent the total number of tumors/group (n=38-42 rats/group); tumors represent macroscopic lesions > $1mm^2$

Interestingly, the HFF group had a similar number of large tumors as the HFB group (10 and 11, respectively), followed by the HFC and LFC groups. The total number of tumors among the groups at week 24 was in the following order: HFB>HFC>LFC>HFF (Table 5.4). These results were not significantly different.

Distribution of tumors along the length of the colon is shown in Table 5.5. It is apparent that of all tumors, a majority of them were in the region B and C representing the 4 to 12 cm region from the rectal end. The LFC group had a higher proportion of total lesions in the regions A and D.

## 5.4 Discussion

The main objective of the present investigation was to determine the ability of preneoplastic stages to respond to the growth modulating effects of dietary lipids, varying in fatty acid composition. Morphological determinants of the different stages of colon carcinogenesis used in the present research were: ACF of different growth features; microadenomas; and macroscopic adenomatous lesions. Dietary lipid intervention was carried out 12 weeks after the last treatment with azoxymethane. This approach allows assessment of the growth modulating effect of a specific diet on preneoplastic lesions already established at different developmental stages. The dietary lipids chosen were a low and high corn oil diet (LFC and HFC respectively), a high fish oil diet (HFF), and a high beef tallow diet (HFB). Enumeration of the number and growth features of ACF, microadenomas and adenomatous lesions before and after dietary intervention enables the quantification of alterations in the number of lesions with various growth features over time.

**Table 5.5 Distribution of Tumors Along the Length of the Colon in Male F344 Rats as Affected by HFF, HFC, LFC and HFB Diets[1]**

| | Colon Segment[2] | | | |
|---|---|---|---|---|
| Group | A | B | C | D |
| HFF | 10.0 | 33.3 | 40.0 | 13.3 |
| HFC | 15.0 | 42.5 | 32.5 | 10.0 |
| LFC | 26.6 | 16.6 | 36.6 | 20.0 |
| HFB | 11.1 | 51.1 | 24.4 | 13.3 |

1. Values are expressed as percentages
2. Location from the rectal end in cm: 0-4 (A), 4-8 (B), 8-12 (C), >12 (D)
3. Total number of tumors in each group are as follows: HFF, 29; HFC, 41; LFC, 31; HFB, 45.

The main findings of the present study are: 1) the HFF diet retarded the appearance of tumors by mechanisms which differ from the LFC diet; 2) the HFF diet exerted a growth stimulatory effect on primal ACF and an inhibitory effect on MA; 3) the HFC and HFB diets stimulated the appearance of MA and tumors by affecting different stages of colon carcinogenesis; and 4) all fat types, regardless of their fatty acid composition, did not modulate the number of large tumors among the groups. Enumeration of ACF and their growth features before and after dietary intervention has provided important insight into the disease process, as affected by dietary lipids. The ACF system has been used extensively to study initiators and modulators of colon carcinogenesis (Bird, 1995; Bird et al., 1996; Magnuson et al., 1994; Wargovitch et al., 1996). However, the opportunity to investigate the multistages of colon carcinogenesis employing ACF exhibiting different growth features has been exploited to a limited extent.

The HFB and HFF groups had a higher number of ACF with advanced growth features at both time points (6 or 12 weeks of feeding) than the HFC or LFC groups. The number of ACF with advanced growth features increased in the HFC group only at week 12, indicating that these lesions were growing more slowly than the lesions in the HFF or HFB groups. Evaluation of the number and growth features of ACF revealed that in spite of the fact that HFC and HFB both increased MA and tumors among the groups, these diets possibly exerted growth-enhancing responses by different mechanisms. It was apparent that a higher number of MA appeared within 6 weeks in the HFC group, whereas, an

increase in MA appeared in the HFB group sometime during 6-12 weeks of feeding. This suggested that the rate of conversion of advanced preneoplastic lesions proceeding to MA was more rapid in the HFC group than in the HFB group, or that the growth stimulatory effect of the HFC group was likely exerted on selected advanced preneoplastic lesions leading to the appearance of MA. In the HFB diet group, the appearance of MA required longer duration than in the HFC group, suggesting that HFB exerted a growth stimulatory effect on preneoplastic lesions that were less advanced than those lesions that were affected by HFC. The finding that the HFB diet stimulated the growth of ACF in all growth categories corroborates this notion. The possibility that HFC must be exerting its growth stimulatory effect on a selected population of advanced preneoplastic lesions is based on the observation that after week 12 of dietary intervention, the number of advanced ACF with ≥7 crypt multiplicity did not change notably in this group. In contrast, marked increases were seen in the number of ACF with advanced growth features in the HFC group than were seen in all other diet groups.

The higher number of tumors also reflected the ability of the HFC diet to stimulate microscopic preneoplastic lesions to progress to the MA and tumor stage during the 12 week feeding duration. The diet groups, including the LFC group, did not differ from each other with respect to the number of adenomas and large adenomas. This observation provides additional evidence for the concept that preneoplastic lesions reach a stage at which time they acquire an established phenotype with enhanced growth autonomy, and are not responsive

to dietary manipulation (Bird et al., 1996). Average tumor size was similar among the HFC, HFB and LFC groups, and highest in the HFF group. This reflected the lower number of small tumors in the HFF group, indicating that although the number of tumors in the HFF group was lower, the tumors present were significantly larger. In comparison, the HFC group had the lowest value for average tumor size because of several small tumors along with large tumors resulting in the average tumor size to be smaller than the HFF or HFB groups. Appearance of a higher number of tumors in the HFC and HFB groups suggests that these diets permitted conversion of several MA into more advanced lesions. In contrast, the HFF diet was least permissive. Interestingly, the reduced number of MA in the HFF group at week 12 suggests that this diet not only retards the development of MA into macroscopic lesions, but must also have induced remodelling or regressed these lesions.

In the present study, the HFB and HFF diets stimulated the growth of primal ACF with a subsequent increase in the appearance of MA. In this regard one can argue that the HFF diet was acting as a high beef tallow diet in mitigating a growth stimulatory effect on primal ACF. This finding differs from that of recent studies in which docosaheaxanoic acid (DHA), piroxicam or other inhibitors of cyclooxygenase pathways and eicosanoid synthesis showed an inhibition in the number of ACF within a few weeks of feeding (Cory et al., 1983; Morishita et al., 1997; Periera et al., 1996; Reddy et al., 1987; Reddy et al., 1992). However, in these studies the experimental diets were fed soon after carcinogen injection. Presumably these diets reduced the number and growth of

ACF by affecting early responses elicited by the carcinogen and may be responsible for the appearance of ACF. It is also plausible that the HFF diet exerts a different effect than those effects exerted by DHA or nonsteroidal anti-inflammatory drug feeding.

The change in the distribution of tumors along the length of the colon was intriguing. In the present study, animals fed a low fat diet seemed to have a higher proportion of tumors in the most distal (0-4 cm) and proximal (>12 cm) regions compared to the high fat diets, and this observation corroborated our previous finding (Bird et al., 1996). The HFB and HFC groups had a higher proportion in the 4-8 cm region of the colon than the HFF of LFC groups. These findings indicate that the fatty acid composition of a high fat diet exerts differential effects on specific colonic regions.

The mechanisms by which HFF, HFB or HFC exerted variable responses on the growth of preneoplastic stages remain elusive. Dietary lipids have been implicated in, and demonstrated to be important modulators of a variety of cellular responses at the membrane, as well as nuclear level (Clandinin et al., 1991; Jump et al., 1997). It is possible that the lipid requirements of preneoplastic lesions at specific developmental stages may differ. Primal lesions are less influenced by the linoleic acid content of the lipid than their more advanced counterparts. The HFC diet, as a rich source of linoleic acid, may favor the growth of advanced preneoplastic lesions. The opposite will be true for a HFF diet, which is a rich source of long chain ω-3 fatty acids that compete with arachidonic acid (AA), a metabolic derivative of linoleic acid. The long chain ω-3

fatty acids found in fish oil (DHA and EPA) have been shown to inhibit the cyclooxygenase pathway, and interfere with AA metabolism for prostaglandin synthesis (Cory et al., 1983; Culp et al., 1979). EPA, in particular, replaces AA in cell phospholipids, and gives rise to prostaglandin species ($PGE_3$ series) lower in biological activity than those derived from AA (Culp et al., 1979; Fischer, 1989). Recent studies examining the effect of fish oil on rectal epithelial proliferation also found lower mucosal levels of $PGE_2$ to be associated with a reduction in mucosal proliferation in high-risk individuals for colon cancer (Anti et al., 1994; Bartram et al., 1993). A number of metabolites of AA have been found to be elevated in colonic mucosa harbouring preneoplastic lesions and in colonic tumors (Rao et al., 1996). If AA metabolites are important for tumor growth than how can we explain the presence of large tumors in the colons of rats fed HFF diet? The one possible explanation is that the ability of some tumors to grow well in the colons of rats fed HFF, HFB or LFC diet supports the concept that advanced lesions adapt to adverse growth environments possibly by their ability to produce additional growth modulators or by adapting a pathway leading to enhanced growth autonomy.

It has been reported previously that rats fed a high fish oil diet soon after carcinogen exposure (1-2 weeks) have a lower tumor incidence than rats fed a high corn oil diet (Reddy, 1994). Moreover, tumors present in the colons of rats fed a high fish oil diet had lowers level of 2 series eicosanoids than the tumors present in the high corn oil fed group (Rao et al., 1996). These findings would suggest that: 1) metabolic processes of tumor cells were altered by dietary lipids;

and 2) despite these changes, tumors were able to grow equally well in both groups. Certainly in the present study a high fish oil diet was able to retard the appearance of a majority, but not all, of tumors. These findings illustrate that biological heterogeneity exists among tumors, and that the ability to adapt to negative growth modulation is key in the continued survival of affected tumor cells. There are two possibilities by which HFF may exert its effect. One is by inhibiting COX activity. Fish oil is known to inhibit COX, and several inhibitors of COX regress the growth and appearance of adenomas in humans (Giardiello et al., 1993, Sandler, 1996). The second possibility is that the HFF diet is driving selected lesions to a potentially differentiated phenotype, where they are unable to maintain their ability to clonally expand. This suggestion is based on the fact that ω-3 fatty acids are present in large amounts in differentiated tissue, such as nerve tissue, and has been associated with differentiation (Jump et al., 1997). The second proposed explanation warrants further investigation.

Several studies have reported the effect of a diet high in fish oil on colon carcinogenesis to be similar to that of a low fat corn oil diet (Minoura et al., 1988; Reddy & Maruyama, 1986; Reddy & Sugie, 1988; Reddy et al., 1991). However, few of these studies have examined the implications of a high fish oil diet on the precursor lesions leading to this preventative effect, and the mechanism(s) behind it. In the present study, tumor outcomes in the LFC and HFF groups were similar, yet analysis of the growth features of ACF and MA in the LFC and HFF groups revealed they produced the same tumor outcome by distinctly different mechanisms. Therefore, it can be concluded that a high fat fish oil diet does not

act like a low fat diet, and that it may have therapeutic value in high-risk individuals harbouring preneoplastic lesions of different dimensions.

The ability to dissect out the disease process and the effect of dietary lipids on the different stages of colon cancer is based on the concept that an increase or decrease in the number of lesions with specific growth features is due to the fact that the diet was able to stimulate or retard the growth of precursor lesions. It is important to note that an increase in the number of a specific group of lesions may also reflect that further progression of these lesions to advanced stages was retarded, resulting in their accumulation in the colon. However, this interpretation will only hold if the appearance of subsequent advanced lesions is retarded. This could be the situation in the HFF-fed rats, resulting in an accumulation of preneoplastic lesions with advanced growth features, i.e. MA or advanced ACF, because their transition to more advanced adenomatous stages was impeded, and a decline in their total number was seen. Alternatively, if the diet is exerting an effect only on selected lesions, it is difficult to predict the disease outcome based on the number and growth features of ACF alone preceding the appearance of MA or tumors, as was noted in the HFC group.

The main contributions of the present study are that that dietary lipids with specific fatty acid composition exerted a growth enhancing or inhibiting effect on the different preneoplastic stages in a selective and differential manner. Each diet exerted distinct responses. The most significant finding of the present study is that HFF potentially retarded as well as regressed advanced preneoplastic

lesions and MA. However, the HFF diet was unable to retard the growth of established lesions that continued to rapidly progress and form tumors of substantial size. In particular, the findings of this study raise important questions as to the role of ω-3 fatty acids in the growth of tumors, which may have invaluable implications for individuals with a high risk for developing colon cancer. In order to identify and understand the underlying mechanisms critical to the progression of selected preneoplastic lesions to more advanced stages of growth, it is essential to examine all stages of carcinogenesis. Exploring the cellular and molecular mechanisms by which these lipids exert their responses may provide insight in to their role in the etiology and prevention of colon carcinogenesis, and their value as a therapeutic agent.

# CHAPTER 6

# A COMPARATIVE STUDY OF DIETARY LIPIDS AND PIROXICAM ON THE GROWTH OF ACF AND COLONIC TUMOR INCIDENCE

## 6.1 Introduction

It has been demonstrated that high fat diets varying in fatty acid composition affect different stages of colonic tumor development (Chapter 5). A diet high in beef tallow or corn oil appears to increase tumor incidence, but has limited effects on the growth of ACF. Whereas, a diet high in fish oil strongly promotes the progression of preneoplastic stages, but has an inhibitory effect on tumor growth compared to the other dietary lipids.

Many studies have speculated as to the possible mechanisms underlying the effects of dietary lipids on tumor growth. One theory suggests that the production and biological effects of prostaglandins are involved in tumor development and progression. This is supported by evidence that colonic tumors have increased expression of cyclooxygenase (COX) leading to increased production of $PGE_2$. From this, one could assume that prostaglandin production would be critical to the growth of tumors in a high fat corn oil diet, since it would provide the highest source of substrate for COX, AA formed from linoleic acid. Since it is known that long chain ω-3 fatty acids such as EPA and DHA compete with AA for COX, and may also directly inhibit COX activity, a decreased production and biological activity of prostanoids may contribute to the tumor-preventive effects of fish oil. This theory is substantiated by the fact that there is

increased $PGE_2$ production in colonic mucosa and tumors from animals fed a high corn oil diet compared to a high fish oil diet (Rao et al., 1996).

Much attention has been paid to the effects of aspirin and other NSAIDS as inhibitors of colonic cancer. COX appears as two isoforms. COX1, which is responsible for maintaining basal physiological levels of eicosanoids, and COX2, which is the more inducible form. Studies using piroxicam, an NSAID which inhibits both isoforms, demonstrated a dramatic decrease in tumor incidence and prostaglandin production in AOM-treated rats (Liu et al, 1995; Reddy et al., 1987). One study also reported that piroxicam inhibits the growth of ACF (Pereira et al., 1996). It should be noted that, to the author's knowledge, all studies investigating the role of piroxicam as a chemopreventive agent in animal models added the compound to a low-fat corn oil diet (AIN-76). This raises the question as to whether the inihibitory effects of piroxicam would be as potent in combination with a diet high in linoleic acid? This would also address the notion that prostaglandins are indeed responsible for the tumor-promoting effects of a high corn oil diet. Comparatively, if decreased prostaglandin production is the mechanism underlying the decreased tumor incidence of a diet high in fish oil, then it should act similarly to a diet containing piroxicam. It is also unclear as to which stage of preneoplastic and neoplastic development piroxicam exerts its inhibitory effects.

Therefore, the objective of the present investigation was to assess the effects of piroxicam, in conjunction with a high fat corn oil diet, on the growth of established preneoplastic lesions, microadenomas and tumors in comparison

with other high fat diets. In addition, to observe if the effects of fish oil and piroxicam on ACF and tumors are similar, due to decreased COX activity. The dietary interventions (HFC, HFB, HFF, HFP) were carried out 10 weeks after the final AOM injection after feeding a standard low-fat control diet.

## 6.2 Materials and Methods

*Animal Care and Experimental Diets*

Male Fischer 344 rats were purchased from the Central Animal Care Facility (University of Manitoba) and acclimatized. All animals were approximately 90-100 g at the time of injection. The conditions of animal care were according to the Canadian Council of Animal Care as described in Chapter 4. Experimental diets were formulated on the basis of the AIN-76A diet (American Institute of Nutrition, 1977) containing 5% (by weight) corn oil (Appendix B). All test lipids were given as an additional 18% by weight in the diet. Piroxicam was supplied as 150 ppm in the high-fat corn oil diet (Table 6.1). Descriptions of fatty acid composition of dietary lipids and experimental diets are supplied in Appendix C. The stock of beef tallow and corn oil, along with the prepared diets, were kept at 4°C. Menhaden oil was provided by Zapata Protein Inc, delivered weekly, and stored at -20°C until used. Prepared fish oil diet was tightly sealed and stored at -20°C to minimize oxidation. All diets were prepared twice weekly.

*Experimental Design*

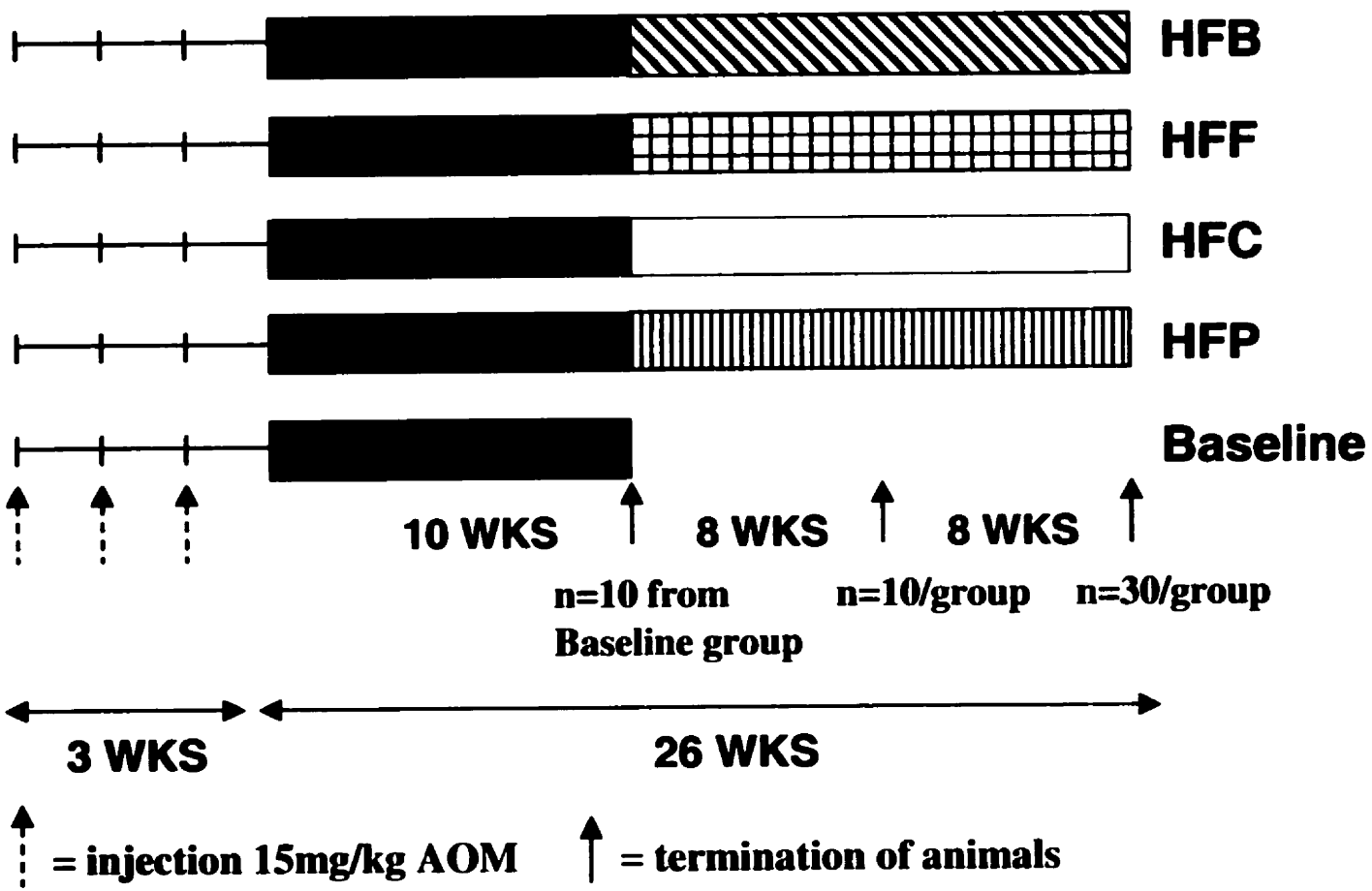
A summary of the study protocol is detailed in Figure 6.1. Male weanling F344 rats were injected three times with AOM in fresh saline (15 mg/kg/wk).

**Table 6.1. Composition of Experimental Diets[1]**

| Component | HFP[2] | HFC | HFF | HFB |
|---|---|---|---|---|
| Casein | 23.0 | 23.0 | 23.0 | 23.0 |
| Corn Starch | 33.8 | 33.8 | 33.8 | 33.8 |
| Dextrose | 8.5 | 8.5 | 8.5 | 8.5 |
| Corn oil | 23.0 | 23.0 | 5.0 | 5.0 |
| Test oil | 0.0 | 0.0 | 18.0 | 18.0 |
| Piroxicam (ppm) | 150.0 | 0.0 | 0.0 | 0.0 |
| Cellufil | 5.9 | 5.9 | 5.9 | 5.9 |
| DL-Methionine | 0.3 | 0.3 | 0.3 | 0.3 |
| Choline bitartrate | 0.2 | 0.2 | 0.2 | 0.2 |
| AIN-76A mineral mix | 4.1 | 4.1 | 4.1 | 4.1 |
| AIN-76A vitamin mix | 1.2 | 1.2 | 1.2 | 1.2 |

1. Values represent the percentage composition
2. Abbreviations are as follows: HFP, high fat corn oil + piroxicam; HFB, high fat beef tallow; HFC, high fat corn oil; HFF, high fat fish oil.

Figure 6.1 Schematic representation of the experimental protocol (broken arrows designate injections with AOM, solid arrows indicate termination of animals). Animals were fed standard lab chow during carcinogen injection, and then given free access to control diet (LFC) for 10 weeks. At this time, 10 animals were killed to provide baseline data. The remaining animals were randomly divided to four diet groups HFC, HFB, HFP or HFF. After 8 weeks of feeding (18 weeks after the last AOM injection), 10 animals per group were terminated. After 16 weeks of feeding (26 weeks after the last AOM injection), the remaining animals were terminated for all dietary groups (approximately 30 rats/group).


HFB
HFF
HFC
HFP
Baseline
10 WKS
8 WKS
8 WKS
n=10 from Baseline group
n=10/group
n=30/group
3 WKS
26 WKS
= injection 15mg/kg AOM
= termination of animals

Animals were given free access to laboratory chow over the duration of the injection period. One week after the third and final injection, all animals were placed on the low-fat corn oil diet (LFC, control). After 10 weeks of LFC feeding, 10 animals were killed by $CO_2$ asphyxiation, and their colons were assessed for ACF and adenomatous lesions. This was considered the baseline group and represented baseline values of ACF prior to diet intervention. The remaining animals were randomly subdivided into four diet groups, HFC, HFB, HFF and HFP, respectively (Figure 6.1). After eight weeks of feeding the experimental diets, 10 animals per group were terminated, and their colons assessed for tumors and ACF. The remainder of the animals was terminated after week 16 of feeding (26 weeks after the last carcinogen injection). Tumor incidence was assessed for all animals in each group. Ten animals per group were designated for ACF analysis.

*Quantification of ACF*

Enumeration of the total number and crypt multiplicity of ACF per colon was performed as described in Chapter 3.

*Assessment of Tumors*

Tumor parameters were assessed according to the methods outlined in Chapter 3.

*Statistical Analysis*

Statistical analysis of the data was accomplished using SAS statistical software for microcomputers as described in Chapter 3. Differences among the groups were determined by analysis of variance in combination with Duncan's

Multiple Range Test at $P \leq 0.05$. Differences in tumor incidence were determined by $\chi^2$ analysis.

## 6.3 Results

At the time when dietary intervention was initiated (after 12 weeks of feeding the control diet), animals had an average of 483.7±ACF/colon. The crypt multiplicity of ACF ranged from 1 to 6 or more crypts/focus (Table 6.2). These results were used as baseline data prior to diet intervention and are represented as the Baseline group.

After 8 weeks of feeding the experimental diets, the colons of the animals were examined for preneoplastic changes and were compared with the changes that were present at the initial time of intervention. After 8 weeks of diet intervention, the HFB group had the highest average number of total ACF/colon, followed by HFF, HFC and HFP, although the differences were not found to be significant (Table 6.2). The HFB and HFF groups had the highest average crypt multiplicity (CM) compared to the other diet groups. There was a trend for the HFB group to have the highest number of primal and intermediate ACF (ACF1-ACF6), although the HFF group had the highest number of advanced ACF with ≥7 crypts/focus ($P \leq 0.05$). The HFP and HFC groups behaved similarly, and overall the HFP had the lowest number of ACF in all growth classifications measured, with the exception of ACF with 4-6 crypts. Among the 10 rats examined in each group, there were 4-8 tumors per group (results not shown). After 16 weeks of feeding the HFC group exhibited the highest number of total ACF. Interestingly a slight decrease in the total number of ACF was observed for

**Table 6.2 Enumeration of ACF Growth Characteristics in Male F344 Rats After 8 Weeks of Diet Intervention with HFF, HFC, HFP and HFB Diets** [1,2,3,4]

| Diet Group | Total ACF | CM | ACF 1-3 | ACF 4-6 | ACF ≥ 7 |
|---|---|---|---|---|---|
| HFF | 416.1±73.0 | 2.6±0.2 [a] | 328.5±58.4[ab] | 81.4±23.3 | 9.6±3.8[a] |
| HFC | 400.2±85.7 | 2.5±0.2 [ab] | 327.2±67.2[ab] | 65.7±25.9 | 6.9±5.9 [ab] |
| HFP | 381.3±102.1 | 2.5±0.6 [ab] | 305.4±85.6[b] | 69.6±25.7 | 5.9±2.4 [ab] |
| HFB | 438.0±72.3 | 2.6±0.2 [a] | 338.8±38.4[ab] | 91.0±38.1 | 8.0±2.0[ab] |
| Baseline | 483.7±145.0 | 2.3±0.2 [b] | 407.7±115.0[a] | 70.8±33.2 | 4.3±3.7[b] |

1. Values are expressed as mean ± SD
2. Values in the same column not sharing a common superscript are significantly different ($P \leq 0.05$)
3. Animals were terminated after 8 weeks of diet intervention, or 18 weeks after the last injection of AOM
4. Abbreviations are as follows: ACF, aberrant crypt foci; CM, average crypt multiplicity/group; HFB, high fat beef tallow; HFC, high fat corn oil; HFF, high fat fish oil; HFP; high fat corn oil + piroxicam, Baseline, levels before diet intervention

**Table 6.3 Enumeration of ACF Growth Characteristics in Male F344 Rats After 16 Weeks of Diet Intervention with HFF, HFC, HFP and HFB Diets[1,2,3]**

| Diet Group | Total ACF | CM | ACF 1-3 | ACF 4-6 | ACF ≥ 7 |
|---|---|---|---|---|---|
| HFF | 307.63±87.7[b] | 3.1±0.3[a] | 205.0±57.8[c] | 88.8±31.1 | 12.2±8.5 |
| HFC | 407.2±95.7[ab] | 2.7±0.2[b] | 310.8±67.6[b] | 78.5±25.0 | 13.2±8.2 |
| HFP | 341.5±65.2[b] | 2.6±0.1[b] | 271.2±59.7[bc] | 62.8±15.4 | 6.2±2.9 |
| HFB | 303.4±88.6[b] | 3.0±0.3[a] | 214.6±64.0[bc] | 74.2±28.7 | 34.4±44.4[a] |
| Baseline | 483.7±145.0[a] | 2.3±0.2[c] | 407.7±115.0[a] | 70.8±33.2 | 4.3±3.7 |

1. Values are expressed as mean ± SD
2. Values in the same column not sharing a common superscript are significantly different ($P \leq 0.05$)
3. Animals were terminated after 16 weeks of diet intervention, or 26 weeks after the last injection of AOM

all groups except the HFC group. Despite a lower number of total ACF, the average crypt multiplicity increased in all groups. The total average crypt multiplicity was in the order of HFF>HFB>HFC>HFP, and there were significant differences among the groups (Table 6.3). The effect of HFP on the number of ACF with more advanced growth features was evident as it was distinctly lower than the other groups. The HFB group had a dramatically higher number of ACF with more than 7 crypts compared to the other groups ($P \leq 0.05$). Examination of the number of ACF with different growth features revealed that HFF, HFB and HFC groups had increases in the number of ACF in all growth categories, whereas the HFP group had minimal increases, particularly in ACF with higher crypt multiplicity (>4 crypts/focus).

All animals were also examined for macroscopic lesions and MA. Tumor incidence was highest in the HFB group compared followed by HFC, HFF and finally the HFP group had only 30% tumor incidence. Interestingly, both average tumor size/group and average tumor size/tumor bearing rat was in the order of HFF>HFB>HFP>HFC, although there were no significant differences among the groups. Total tumor burden was also highest in the HFF group followed by HFB, HFC and finally HFP was the lowest. The HFC group had the highest number of MA (9), and the HFP group had the lowest with only one MA (Table 6.4).

All tumors were further categorized by size into small, medium and large tumors as done previously (Chapter 5). The HFP group had the lowest number of small tumors, while the HFB had the highest number and percentage of small tumors (Table 6.5). Interestingly, the HFP had a similar number of medium

**Table 6.4 Tumor Incidence Parameters in Male F344 Rats Fed HFF, HFB, HFC and HFP diets [1,2,3]**

| | HFF | HFC | HFP | HFB |
|---|---|---|---|---|
| Total number of rats | 43 | 42 | 43 | 42 |
| Tumor incidence | 56% | 57% | 30%[a] | 69% |
| Average tumor size/group ($mm^2$) | 17.6±20.9 | 13.2±14.6 | 13.8±10.6 | 14.8±17.7 |
| Tumor multiplicity | 1.48±0.90 | 1.83±1.19 | 1.15±0.38 | 1.89±1.65 |
| Average tumor size/tumor bearing rat ($mm^2$) | 16.9±18.4 | 13.5±14.1 | 14.15±10.7 | 14.7±14.5 |
| Tumor burden | 28.1±35.2 | 24.0±22.0 | 15.9±11.4 | 24.7±27.7 |
| Total MA/group[4] | 2 | 9 | 1 | 5 |

1. Values are expressed as mean ± SD
2. Values in the same row not sharing a common superscript are significantly different ($P \leq 0.05$)
3. Animals were terminated after 16 weeks of diet intervention, or 26 weeks after the last injection of AOM
4. Microadenomas representing microscopic lesions ≤ $1mm^2$

**Table 6.5 Classification of Tumor Size in Male F344 Rats After 16 Weeks of Intervention with HFF, HFC, HFP and HFB Diets[1,2]**

| | Diet | | | |
|---|---|---|---|---|
| Tumor Size | HFF | HFC | HFP | HFB |
| Small | 58.3 (21) | 65.1 (28) | 46.6 (7) | 68.9 (31) |
| Medium | 22.2 (8) | 13.9 (6) | 40.0 (6) | 11.1 (5) |
| Large | 19.5 (7) | 21.0 (9) | 13.3 (2) | 20.0 (9) |
| Total Tumors[3] | 36 | 43 | 15 | 45 |

1. Values are expressed as percentage of tumors, with actual number in parentheses
2. Categories are as follows: small, <10 $mm^2$, medium, <20 $mm^2$, large, >20 $mm^2$
3. Values represent total number of tumors/group (n = 40-43 rats/group); tumors represent macroscopic lesions > 1$mm^2$

tumors compared to the other groups, and a majority of the total tumors found in that group were mid-sized (40%). The proportion of large tumors was similar among the HFF, HFC and HFB groups, with HFP being the lowest. The total number of tumors per group was in the descending order of HFB>HFC>HFF>HFP. The HFP group had a significantly lower number of tumors compared to the other high-fat diets ($P \leq 0.05$). Distribution of tumors along the length of the colon is presented in Table 6.6. The majority of tumors from all groups were found in the mid-portion of the colon represented by regions B and C (4 to 12 cm from the rectal end). The HFP group had the lowest percentage of tumors in region A, but the highest percentage in region D compared to the other diet groups.

## 6.4 Discussion

The present study was conducted to determine the growth-modulating effects of a purported tumor inhibitor (piroxicam) when given in conjunction with a tumor-promoting high-fat corn oil diet. The experimental approach taken was similar to that used in the previous study (Chapter 5). Briefly, dietary intervention was carried out 10 weeks after the last treatment of AOM. At this time, the injected colons harbour preneoplastic lesions of varying developmental stages. Assessment of the number of ACF and their crypt multiplicity before and 16 weeks after feeding the experimental diets makes it possible to determine which stage in particular is responding to the dietary treatment. The main findings of the present study are as follows: 1) Piroxicam inhibited the appearance of advanced ACF and tumors without decreasing the total number of ACF; 2) A diet

**Table 6.6 Distribution of Tumors Along the Length of the Colon in Male F344 Rats as Affected by HFF, HFC, HFP and HFB Diets[1]**

| Group | Colon Segment[2] | | | |
|---|---|---|---|---|
| | A | B | C | D |
| HFF[3] | 20.0 | 31.4 | 45.7 | 2.9 |
| HFC | 19.0 | 35.7 | 35.7 | 9.6 |
| HFP | 6.7 | 33.3 | 40.0 | 20.0 |
| HFB | 18.1 | 41.0 | 27.3 | 13.6 |

1. Values are expressed as percentages
2. Location from the rectal end in cm: 0-4 (A), 4-8 (B), 8-12 (C), >12 (D)
3. Total number of tumors in each group are as follows: HFF, 36; HFC, 43; HFP, 15; HFB, 45.

high in fish oil does produce a lower tumor incidence compared to the other high fat diets, but is not as effective as piroxicam; 3) The HFB and HFC diets had similar tumor parameters, but different effects on the growth of ACF with higher crypt multiplicity.

Numerous studies have reported the tumor-inhibiting effects of piroxicam in carcinogen-induced colon cancer. The present study is, to the author's knowledge, the first to explore the effects of piroxicam in conjunction with a diet known to promote tumor growth, in this case a diet high in corn oil. It is apparent that piroxicam had a distinct effect on specific stages of carcinogenesis and regions of the colon. The total number of ACF at both termination points (8 and 16 weeks of diet intervention) in animals fed piroxicam was not different from the other high-fat diets. However, there was a trend for the HFP group to have the lowest number of intermediate and advanced ACF compared to the other groups. This suggests that piroxicam has a more inhibitory effect on the growth of advanced compared to newly emerging preneoplastic lesions. These findings may have practical implications for colon cancer prevention as it has been demonstrated that advanced ACF are more autonomous, and therefore, more resistant to growth modulation.

The distal region of the colon was more sensitive to the inhibitory effects of piroxicam, as the lowest proportion of tumors was found in this region compared to the other diet groups. In addition, the HFP group also had the highest proportion of tumors in the proximal region of the colon compared to the other high-fat diets. Although the total number of tumors in the HFP group was

low, the tumors that were able to overcome the inhibitory effects of piroxicam were of considerable size. The HFP group had the lowest percentage of small tumors (46.6%) and highest percentage of medium tumors (40.0%) compared to the other diet groups. Further analysis of the data revealed that the largest tumors from the HFP group were all found in the proximal region of the colon. In addition, HFP had a slightly higher average tumor size/group and tumor size/tumor bearing rat than the HFC group. It was also interesting that the actual number of medium tumors from the HFP diet was not different from the HFB and HFC groups, considering the total number of tumors in the was at least two-thirds lower. These findings suggest preneoplastic lesions that were able to overcome the inhibitory effects of piroxicam to form tumors were just as large or larger than tumors found in other diet groups, and therefore may possess a more aggressive phenotype compared to those lesions that were not.

It has been suggested that the decrease in tumor incidence from animals fed a diet high in fish oil is due to a decreased production of $PGE_2$ by the COX enzymes. From this, one would expect preneoplastic lesions and tumors to respond in a similar manner to fish oil and the COX inhibitor piroxicam. However, the findings of the present study indicate that this is not quite the case. The HFF diet appears to have an intermediate inhibitory effect on tumor growth, as the total number of tumors was lower than the HFC and HFB groups, and yet still two-fold higher compared to the HFP group. Piroxicam is a synthetic COX inhibitor, while fish oil affects prostaglandin production through biological mechanisms such as competition with other fatty acids, and inhibition of COX by

DHA. The HFF group had a higher number of ACF>4 crypts/focus at both time points compared to the HFP group. The effect of fish oil to stimulate the growth of ACF with higher crypt multiplicity was comparable to that observed in the previous study (Chapter 5). In addition, the HFF group had the highest average tumor size/group and tumor burden compared to the other diet groups, which was also seen in the previous study.

The HFF group also had similar number of medium and large-size tumors compared to the HFB and HFC groups. Therefore, these findings are consistent with the conclusion that a diet high in fish oil does have different effects on the growth of cancerous lesions depending on their stage of development, and tumors that did develop were able to grow rapidly and reach a considerable size. One difference between the present and the previous study (Chapter 5), was that the number of MA was much lower in the present study for all groups, specifically for the HFF group. This could be explained by the longer feeding duration of the experimental diets (16 weeks vs. 12 weeks), which may have allowed a higher proportion of MAs to progress and form tumors by the final termination point.

The HFC and HFB diets demonstrated once again to be effective tumor-promoting agents. However, they appear to accomplish this result by eliciting different growth responses in ACF. Enumeration of ACF growth characteristics after 8 weeks of feeding the experimental diets revealed that the total number of ACF was similar between the groups, although the number of intermediate ACF (4-6 crypts/focus) was slightly higher in the HFB group. After 16 weeks of feeding, the number of intermediate ACF was equal between the groups

however, the number of large ACF was over 2-fold higher in HFB compared to HFC. Interestingly, the two groups were quite similar in terms of tumor incidence parameters including tumor distribution and tumor size. It should be noted that the HFB group did have a slightly higher tumor incidence and average tumor size/group. Although there was a smaller number of advanced ACF in the HFC group compared to HFB, a high proportion of those lesions must have progressed to form tumors in order to produce their similar tumor outcome. Whereas, the HFB diet had a more general growth stimulating effect on both intermediate and advanced preneoplastic lesions leading to an increased tumor outcome. These findings demonstrate that although beef tallow and corn oil are tumor promoting, these lipids affect different stages of carcinogenesis leading to the same end result.

In summary, the present study suggests that the growth requirements of ACF for fatty acids and their metabolites may differ depending on their stage of development (primal vs. advanced). Furthermore, that piroxicam + HFC dramatically decreased tumor outcome compared to HFC and HFF suggests that COX may play a critical role in the progression of advanced ACF to form tumors. These findings also suggest that the potential mechanism by which fish oil may decrease colon tumor incidence may be mediated via another pathway, or in addition to a decrease in COX activity. The cellular and molecular mechanisms behind the growth responses of ACF in specific dietary environments need to be further elucidated.

# CHAPTER 7

# A SEMIQUANTITATIVE ASSESSMENT OF TGF-α, EGFR, AND COX GENE EXPRESSION IN COLONIC MUCOSA AND TUMORS AS AFFECTED BY DIETARY LIPIDS AND PIROXICAM

## 7.1 Introduction

The proposed, and well accepted, adenoma-carcinoma sequence in the progression of colon cancer emphasizes the role of altered expression of specific target genes. Investigation of modified gene expression patterns between colonic mucosa and tumors may be critical in identifying and treating the disease before tumor development occurs. The increased expression of EGFR has been reported as a potential indicator of increased malignant potential in human colonic tumors (McLeod & Murray, 1999). It has also been demonstrated that increased expression and activation of EGFR by TGF-α leads to increased expression of COX2 in colon cancer cells (Coffey et al., 1997). The signaling intermediates providing the connection between these two events is likely the ERKs, as they have been shown to activate nuclear transcription factors in response to EGFR activation (Brunet & Pouyssegur, 1997). Inhibition of the up-regulation of COX2 gene expression has been suggested as an attractive therapeutic target for colon cancer prevention (Eberhart et al., 1994). It is not known if an association exists among these signaling elements in the progression of colon cancer, as they have yet to be compared in colonic mucosa and tumors in the same study.

As demonstrated in the preceding chapters, diets high in beef tallow and corn oil increase tumor incidence compared to a diet high in fish oil in carcinogen –treated rats. The increased production of prostaglandins through COX2 has been suggested as a potential mechanism responsible for the tumor-promoting effects of a high-corn-oil diet. This may be true as the addition of piroxicam, a COX inhibitor, to a high-corn-oil diet proved to be most effective in reducing colon cancer incidence (Chapter 6). Piroxicam acts through competitive inhibition of the enzymes, thereby decreasing prostaglandin production and colon tumor development. However, it is not known if there is altered transcription of the COX genes in response to piroxicam treatment.

Most recently there has been increasing evidence that dietary lipids possess the ability to directly affect gene expression (Clarke & Jump, 1994; Sellmayer et al., 1997). It is therefore possible that the tumor-modulating effects of certain dietary lipids in the colon may be influenced by the altered expression of specific target genes.

Based on the aforementioned evidence, the objectives of the present study were the following: 1) to assess the gene expression of TGF-α, EGFR, COX1, COX2 and ERK-1 in carcinogen-treated colonic mucosa and tumors; 2) to investigate if the expression of these target genes is affected by specific dietary lipids or piroxicam; 3) to determine of a possible relationship exists among these factors in relation to colonic tumor progression.

## 7.2 Materials and Methods

*Experimental Design*

Male weanling F344 rats were injected three times with AOM in fresh saline (15 mg/kg/wk). One week after the third and final injection, all animals were placed on the low-fat corn oil diet (LFC, control). After 10 weeks of LFC feeding, the animals were randomly subdivided into four diet groups, HFC, HFB, HFF and HFP, respectively. The animals were terminated after week 16 of feeding (26 weeks after the last carcinogen injection). Colonic mucosal scrapings and tumor samples were taken. All samples were collected under RNAse free conditions using autoclaved solutions and instruments, placed immediately in liquid nitrogen and stored at -80°C until processed.

*RNA Extraction Procedures*

All reagents and enzymes were obtained from Gibco BRL (Burlington, ON) unless otherwise specified. Extraction of RNA was based on the method developed by Chomczynski and Sacchi (1987). Tumors were placed in 500$\mu$l denaturation solution (4M guanidine thiocycanate, 25mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). The samples were homogenized in a 1.5 ml conical microcentrifuge tube with a tissue grinder pestle (Kontes #749515-000) for 30 seconds. 1/10 volume of 2M sodium acetate (pH 5.2), and 1 volume water-saturated ultra pure phenol with 0.1% hydroxyquinoline (w/w) were added. The samples were vortexed, 1/5 volume of chloroform-isoamyl alcohol (49:1 v/v) was added, then vortexed again and incubated on ice for 40 minutes. The resulting suspension was centrifuged for 60 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 a -80°C overnight to precipitate the RNA. The RNA was recovered by centrifuging at 16,000 x *g* at 4°C for 60 minutes. The RNA pellets obtained were washed by adding 400$\mu$l 80% ethanol, and centrifuged 10 minutes at 16,000 x *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 33 $\mu$l autoclaved ultra pure deionized water. 37U DNAse 1 and 38.9U RNA guard (Pharmacia, Montreal, Quebec) were added to a solution consisting of 40mM Tris, pH 7.5 and 6mM $MgCl_2$, added to the suspended RNA and incubated at 37°C for 60 minutes in a volume of 50 $\mu$l. The DNAse treatment was inactivated by 100mM EDTA, phenol extracted and ethanol precipitated as described previously. The RNA pellet was resuspended in 30 $\mu$l autoclaved ultrapure deionized water. The concentration and integrity was determined by spectrophotometry, by the absorbance at 260 nm and 280 nm respectively.

*cDNA Synthesis by Reverse Transcription*

Reverse transcription was performed on 1 $\mu$g total RNA in a 20 $\mu$l reaction volume according to the method detailed by Gibco BRL. Initially, 1 $\mu$g of RNA, 8 $\mu$l of water and 1 $\mu$l of oligo DT (500 $\mu$g/ml) were combined and heated at 65°C for 10 minutes, and was placed immediately on ice for 5 minutes. After cooling, 1 $\mu$l (39U) of RNA Guard (Pharmacia), 2 $\mu$l of 100 mM DTT, 2 $\mu$l of 5mM dNTP and 5X first strand buffer (Appendix F). The mixture was vortexed briefly. Reverse

**Table 7.1 Description of Primer Sequences and Conditions**

| Primer | Sequence | Product Size (bp) | Conditions (Temp/min) | Cycles |
|---|---|---|---|---|
| EGFR[1] | TAGAAATGGGAGCTGCCGTGTC<br>AGGGTTGCTCACCGCCATTG | 231 | 94 55 72<br>1 1 1 | 32 |
| TGF-α[1] | CTTTGTGTTGGCCCTGGTGAGC<br>TCCAGGCGGAAATCGTCACTTG | 334 | 94 52 72<br>1 1 1 | 31 |
| COX1[2] | TGCATGTGGCTGTGGATGTCATCAA<br>CACTAAGACAGACCCGTCATCTCCA | 450 | 94 52 72<br>1 1 1 | 29 |
| COX2[2] | ACTTGCTCACTTTGTTGAGTCATTC<br>TTTGATTAGTACTGTAGGGGTTAATG | 440 | 94 55 72<br>1 1 1 | 34 |
| ERK-1[1] | GCATCAAACCTACTGTCAGCGCACG<br>TGTACTGAGGCCCCGGAGGATCT | 178 | 94 60 72<br>1 1 1 | 27 |
| β-Actin[3] | GTGGGGCGCCCCAGGCACCA<br>CTCCTTAATGTCACGCACGATTTC | 541 | 94 52 72<br>1 1 1 | 23 |

1. Genebank
2. Prostaglandins, Leukotrienes Ess Fatty Acids, 52: 462-481, 1996
3. Cell Growth Diff., 6:1625-1642, 1995

transcriptase was performed by the addition of 1 $\mu$l (200U/$\mu$l) of M-MLV (Moloney Murine Leukaemia Virus) at 42°C for 2 hours.

*Polymerase Chain Reaction*

The sources of the primer sequences, PCR conditions and size of PCR products are described in Table 7.1. All PCR reactions were carried out using a PTC-100 Thermocycler (MJ Research Inc.). Following cDNA synthesis, 1.6 $\mu$l of the cDNA reaction mix was amplified in a 50 $\mu$l PCR reaction mixture. The reaction mix contained 1.6 $\mu$l of cDNA, 16.96 $\mu$l $H_2O$, 2.5 $\mu$l 10 PCR buffer (Appendix F), 2.5 $\mu$l 2 mM dNTP, 0.75 $\mu$l 50 mM $MgCl_2$, 0.50 $\mu$l 25 pmol 3' and 5' primers for either EGFR, TGF-$\alpha$, COX1, COX2, ERK-1 or $\beta$-actin and 0.1875 (5U/$\mu$l) Taq DNA polymerase. The reaction was mixed gently and overlayed with mineral oil (Sigma), preheated for 2 minutes at 94°C and then for the appropriate number of cycles depending on the primer. All samples were subjected to a final elongation period for 10 minutes at 72°C. At the end of the PCR reaction, 5 $\mu$/ of 10X PCR loading buffer was added (Appendix F), and all samples were stored at -20°C until further analysis.

*Visualization and Quantification of PCR Products*

PCR products were separated on a 2% agarose gel containing 3 ml 10X TBE and 4.5 $\mu$l ethidium bromide in TBE buffer (Appendix F) using the Gibco BRL Horizon 11-14 gel electrophoresis apparatus at 150 volts for 25 minutes. Equal volumes of each sample were loaded (15 $\mu$l), and all primers for one sample were loaded on a single gel to reduce variation within samples. The resulting gels were photographed under UV illumination with polaroid film. The

resulting photographs were scanned using Corel PhotoPaint version 5.0 PC software. The area of the product bands detected (pixels) was calculated using Scion Image software (version 2.0). 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 "housekeeping gene" which is present in equal amounts in all cells (Wong et al., 1994), and has been used previously to quantify PCR products from rat colonic neoplasms (Bird et al., 1997; Yoshimi et al., 1994).

*Statistical Analysis*

Statistical analysis of PCR products was conducted by ANOVA with Duncan's Multiple Range Test or Student's t-test. Six to 9 mucosa samples and 4-6 tumor samples were used for each diet group. A $P$ value of $\leq 0.05$ was considered significant unless otherwise noted.

## 7.3 Results

For each primer, sequential amounts of cDNA were used to ensure sample amounts were within the linear range and to avoid the saturation limit of the Taq DNA Polymerase (Figure 7.1).

*Expression of EGFR*

In colonic mucosa, the HFF group had the highest expression of EGFR, followed by HFP, HFC and finally HFB. Tumors from the HFP group had the highest EGFR expression, while HFB tumors had the lowest expression ($1.56 \pm 0.75$ vs. $0.78 \pm 0.27$ units respectively, $P \leq 0.05$). Comparatively, there was a significant increase in EGFR expression between mucosa and tumor samples

Figure 7.1 Agarose gel analysis of the PCR products for ERK-1 (left) and TGF-α (right) genes. Each proceeding lane represents an increase in the amount of cDNA.


1018
506
396
178 bp ERK-1
1 kb Ladder
1 kb Ladder
TGF-α 334 bp

Figure 7.2 Density of RT-PCR products for EGFR gene expression in colonic mucosa and tumors from rats fed HFF, HFP, HFC or HFB diets for 16 weeks. Values are mean ± SD (bars), and are expressed as a ratio of the area of the product band for β-actin. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).

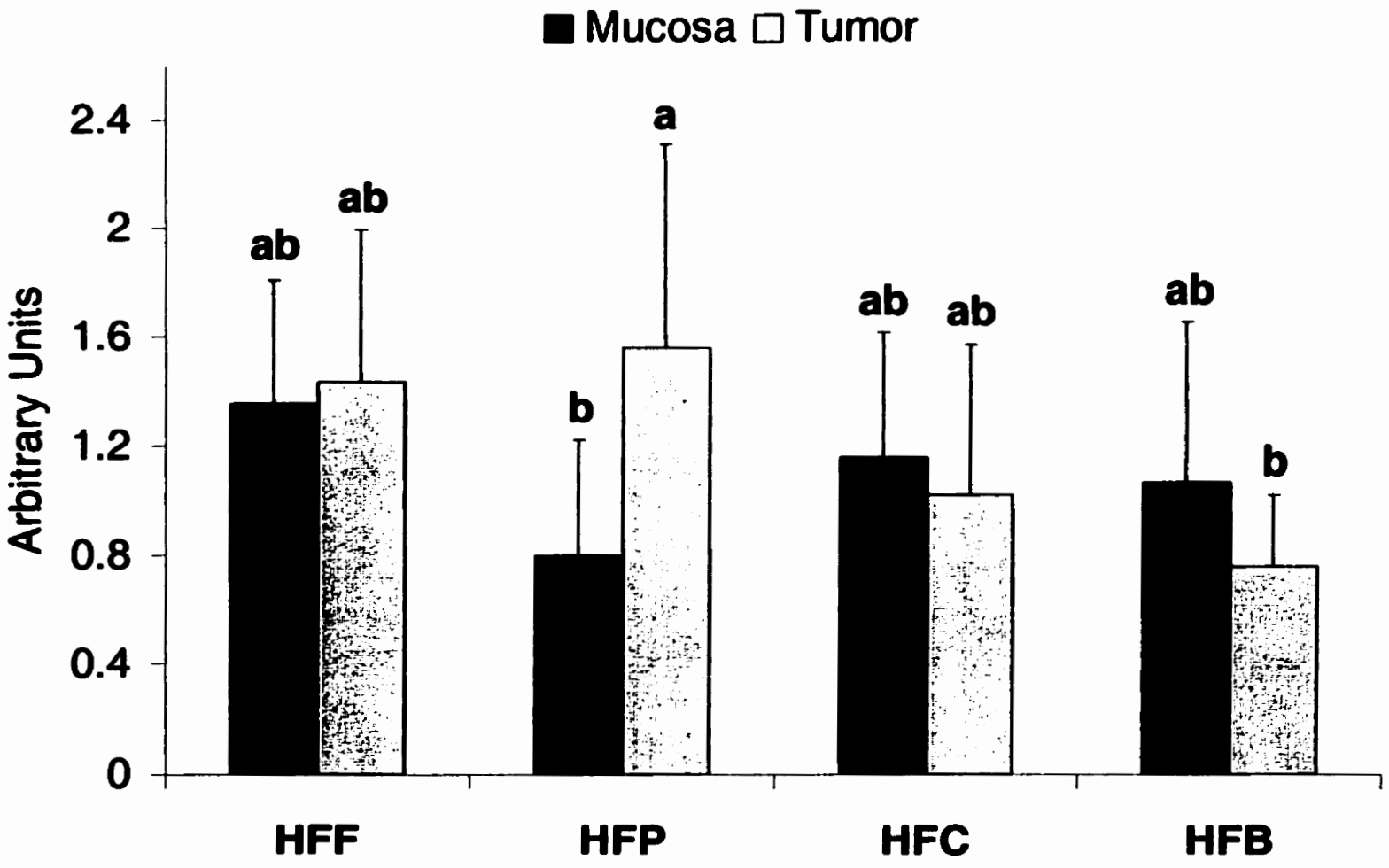
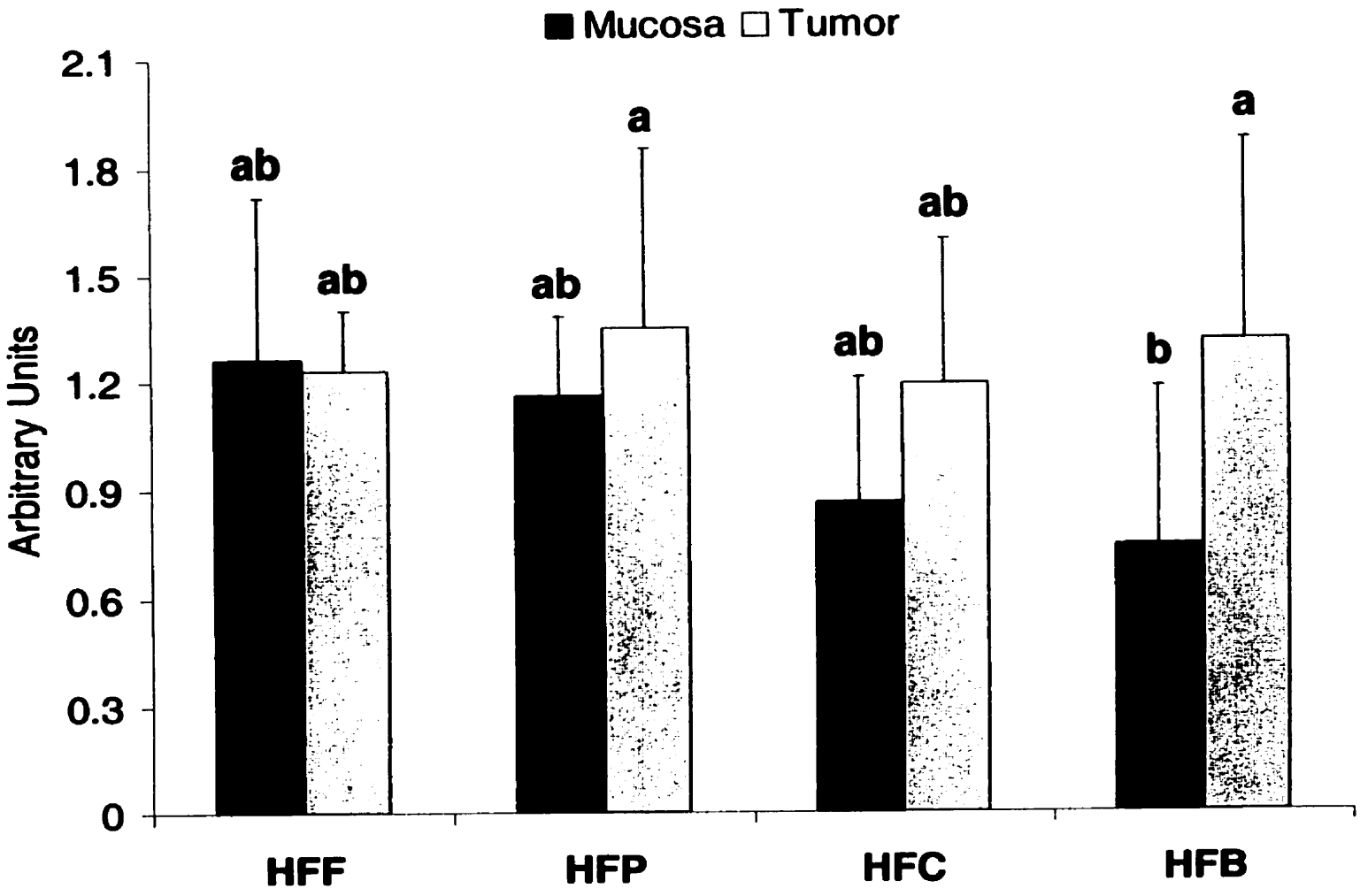

Mucosa
Tumor
Arbitrary Units
2.4
2
1.6
1.2
0.8
0.4
0
ab
ab
b
a
ab
ab
ab
b
HFF
HFP
HFC
HFB

Figure 7.3 Density of RT-PCR products for TGF-α gene expression in colonic mucosa and tumors from rats fed HFF, HFP, HFC or HFB diets for 16 weeks. Values are mean ± SD (bars), and are expressed as a ratio of the area of the product band for β-actin. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).


Mucosa
Tumor
Arbitrary Units
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
ab
ab
ab
a
ab
ab
b
a
HFF
HFP
HFC
HFB

in the HFP group (Figure 7.2). The mucosa and tumor samples from the HFF, HFC and HFB groups were similar in their expression of EGFR.

*Expression of TGF-α*

TGF-α expression among colonic tumors was similar (Figure 7.3). Interestingly, the HFB mucosa was significantly lower in the expression of TGF-α compared to mucosa from the other diet groups, but also compared to HFB tumors ($P \leq 0.05$). The HFP and HFC groups also had a slight increase in tumor TGF-α expression compared to mucosa, while there was no difference in tumor and muocsa from the HFF group.

*Expression of COX1*

There were no significant differences among the groups for the expression of COX1. Mucosal COX1 expression was highest in HFF, followed by HFP, HFC and HFB with a range of $0.39 \pm 0.16$ to $0.13 \pm 0.07$ units, respectively. The expression of COX1 in HFB mucosa was once again significantly lower compared to the other mucosa samples, and also the COX1 expression of HFB tumors ($P \leq 0.05$). Levels of COX1 mRNA when comparing mucosa and tumors from the other diet groups were almost equivalent (Figure 7.4).

*Expression of COX2*

Expression of COX2 in colonic tumors was highest in the HFC group, and lowest in tumors from animals fed the HFB diet (Figure 7.5). There were no differences among the diet groups for either mucosa or tumor gene expression.

Figure 7.4 Density of RT-PCR products for COX1 gene expression in colonic mucosa and tumors from rats fed HFF, HFP, HFC or HFB diets for 16 weeks. Values are mean ± SD (bars), and are expressed as a ratio of the area of the product band for β-actin. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).

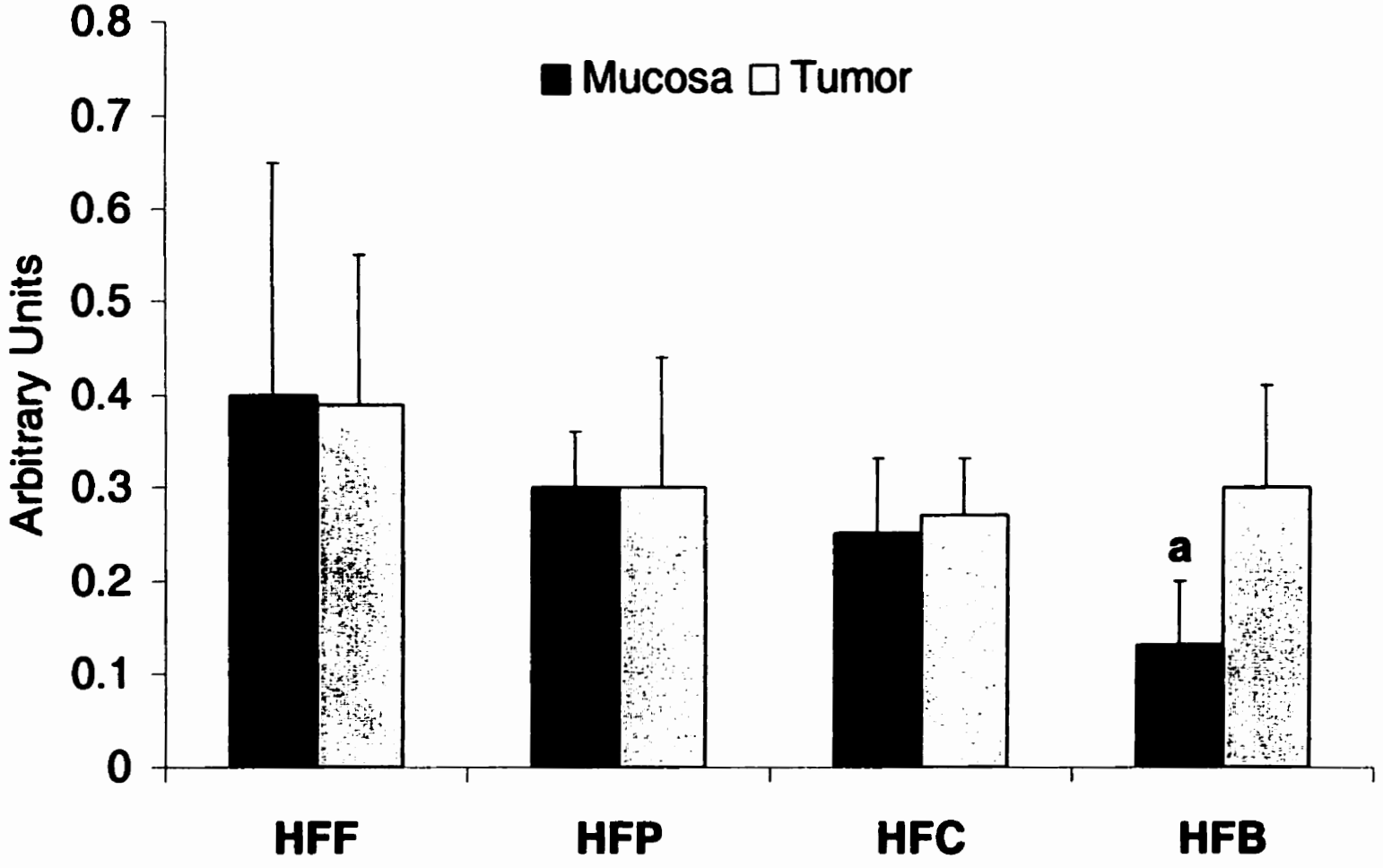
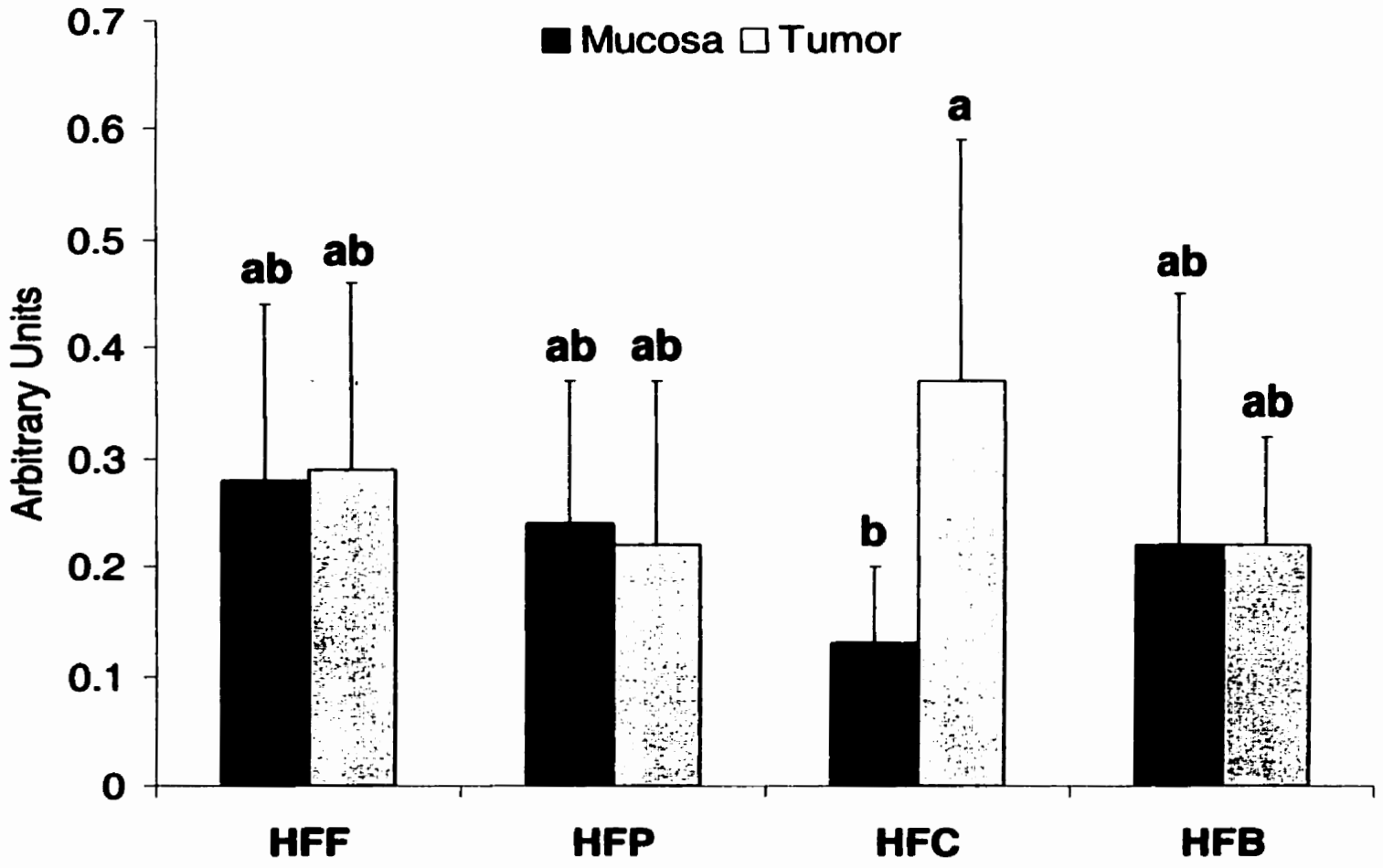

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Arbitrary Units
Mucosa
Tumor
a
HFF
HFP
HFC
HFB

Figure 7.5 Density of RT-PCR products for COX2 gene expression in colonic mucosa and tumors from rats fed HFF, HFP, HFC or HFB diets for 16 weeks. Values are mean ± SD (bars), and are expressed as a ratio of the area of the product band for β-actin. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).


Mucosa
Tumor
Arbitrary Units
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ab
ab
ab
ab
b
a
ab
ab
HFF
HFP
HFC
HFB

There was however, a significant increase in COX2 going from mucosa to tumor in the HFC group (0.13±0.07 vs. 0.37±0.22 units, respectively). There was little change in COX2 expression when comparing mucosa and tumor values from the remaining diet groups.

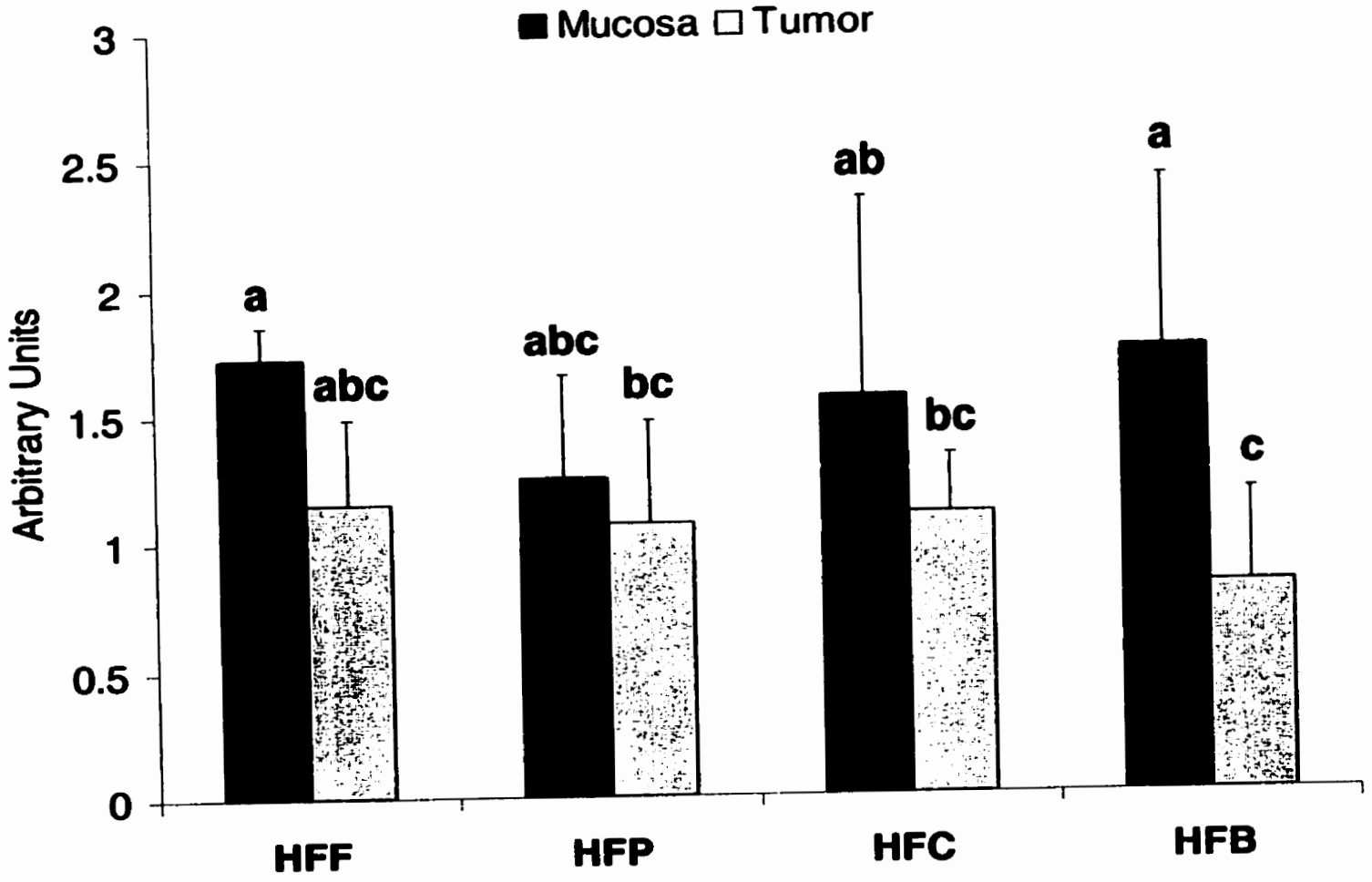
*Expression of ERK-1*

There were no significant diet-induced differences among the groups for either mucosa or tumor samples (Figure 7.6). It is interesting that expression of ERK-1 was higher overall in colonic mucosa compared to tumors, irrespective of the experimental diets. In fact, there were significant decreases in ERK-1 expression when comparing mucosa and tumors for all diet groups ($P \leq 0.05$). The only decrease going from mucosa to tumor was seen in the HFB group (1.74±0.67 vs. 0.81±0.37 units, respectively).

## 7.4 Discussion

The present study was conducted to determine the effect of dietary lipids on the expression of certain target genes (EGFR, TGF-α, COX1, COX2 and ERK-1) implicated in colon carcinogenesis. Furthermore, to determine if there were any differences in the expression of these genes between carcinogen-treated colonic mucosa and colonic tumors harvested from the same diet treatment. The main findings of the present study are the following: 1) There were no diet-induced differences in gene expression for EGFR, TGF-α or ERK-1 in colonic tumors or mucosa among the diet groups; 2) The HFC diet caused a significant increase in tumor COX2 expression compared to mucosa; 3) Tumors

Figure 7.6 Density of RT-PCR products for ERK-1 gene expression in colonic mucosa and tumors from rats fed HFF, HFP, HFC or HFB diets for 16 weeks. Values are mean ± SD (bars), and are expressed as a ratio of the area of the product band for β-actin. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).


Mucosa
Tumor
Arbitrary Units
3
2.5
2
1.5
1
0.5
0
a
abc
abc
bc
ab
bc
a
c
HFF
HFP
HFC
HFB

from the HFB diet had increased levels of TGF-α and COX1 compared to mucosa; 4) The HFP diet caused an up-regulation of tumor EGFR gene expression compared to colonic mucosa.

To the author's knowledge, this is the first comparative analysis of colonic mucosa and tumors integrating the role of gene expression and dietary treatment in tumor development. All samples were taken from animals that had been fed the experimental diets for 16 weeks, as described in Chapter 6. The findings of the previous study revealed that diets high in beef tallow and corn oil had a tumor-promoting effect, the addition of piroxicam to an HFC diet significantly decreased tumor outcome, whereas a diet high in fish oil was less inhibitory. In order to investigate the potential molecular mechanisms behind the differential effects of these specific diets on tumor development, the mRNA expression of TGF-α, its receptor EGFR, the signaling kinase ERK-1 and both COX isoforms was determined. These factors have been individually associated with the initiation and progression of colonic cancer, and recent evidence has suggested a more interdependent relationship exists among them.

The findings of the present study reveal no differences among the diet groups in expression of the genes studied for either colonic mucosa or tumors. Rather, specific genes appear to be altered when comparing the expression between mucosa and tumors within a diet group. It is possible that certain genes are more critical and sensitive in mitigating the growth effects of certain dietary environments. It is interesting to note that in all cases where there was a significant increase in gene expression in colonic tumors for a specific diet, the

levels of that gene found in colonic mucosa were the lowest compared to the other diet groups. It is unknown whether lower expression of a specific gene in colonic mucosa makes it more susceptible to the development of tumors, which are then able to greatly increase the levels of that gene by a feed-back mechanism.

The tumor-promoting effects of ω-6 fatty acids have been associated with an increase in COX2 gene expression and $PGE_2$ production in many epithelial cancers, such as colon, prostate, breast and skin (Hursting et al., 1990). Our findings were similar in that a significant increase in COX2 gene expression was observed only in colonic tumors from the HFC group. It has been shown that the levels of local intracellular $PGE_2$ play a major role in the expression of COX2 (Tjandrawinata & Hughes-Fulford, 1997). One would expect $PGE_2$ production to be much higher in the HFC diet compared to the other experimental diets. Furthermore, the biological features of a tumor as a solid, autonomous mass would perhaps create an increase in the concentration of locally produced $PGE_2$ compared to the levels found in the larger surface area of colonic mucosa. Certain NSAIDS, as well as fish oil, have been reported to decrease levels of both COX1 and COX2 gene expression (Achard et al., 1997; Tjandrawinata & Hughes-Fulford, 1997). Although COX1 and COX2 levels were not increased in colonic tumors compared to mucosa in the HFP and HFF groups of the present study, they were neither decreased.

It is interesting to note that only the HFB group had a significant increase in COX1 expression in colonic tumors compared to the other diets. Moreover,

the HFB group also had the largest comparative increase in tumor TGF-α gene expression. Increased expression of TGF-α has been associated with an increase in $PGE_2$ synthesis (Asano et al., 1997; Tahara et al., 1995). However, these studies were conducted *in vitro* using normal cell types, and it is unclear whether the increase in $PGE_2$ is related to an increase in COX1 and/or COX2 transcription, translation or activity. Current cancer research has focused primarily on the effects of specific PUFA in gene regulation and tumorigenesis. Studies investigating the effects of saturated fatty acids on gene expression have been limited to lipoproteins and various enzymes involved in carbohydrate and lipid metabolism (Jump et al., 1997; Ricketts & Brannon, 1994; Srivastava, 1994). This is somewhat puzzling because of all fat types, only the tumor-promoting effects of a diet high in saturated fat is strongly supported by both epidemiological and experimental studies. To the author's knowledge, the effect of saturated fatty acids on transcriptional regulation of the cellular targets implicated in colon carcinogenesis remains largely unexplored.

The up-regulation of tumor EGFR gene expression only in the HFP group was intriguing. One previous study has reported an increase in EGFR gene expression in colon cancer cells treated with the COX inhibitor indomethacin (Kinoshita et al., 1999). As demonstrated in Chapter 5, piroxicam is a very potent inhibitor of tumor growth, suggesting the tumors that did appear may possess a more aggressive phenotype. Over-expression of EGFR is considered to be a biomarker for increased malignant potential in both breast and colon cancers (McLeod & Murray, 1999). Therefore, the ability of certain advanced

preneoplastic lesions or adenomas to up-regulate EGFR may provide them with a selective growth advantage over other lesions to over-come the inhibitory effects of piroxicam. An increase in EGFR expression has also been associated with an increased in COX2 in colon cancer cells (Coffey et al., 1997). It was interesting to note that there was no concomitant increase in COX2 gene expression in tumors from the HFP group. The inhibitory effects of piroxicam on COX activity may have prevented the feed-back mechanisms required to increase COX2 gene expression.

The finding that tumors had decreased expression of ERK-1 compared to colonic mucosa in all diet groups was surprising. Few, if any, studies have investigated the level of ERK-1 at the nuclear level in colonic tissues, as only the activity and protein levels of this signaling enzyme are typically assessed. Increases in ERK-1 and –2 protein levels in colonic tumors have been reported in numerous studies (Davidson et al., 1999; Licato & Brenner, 1998; Licato et al., 1997). ERK-1 serves as a critical communication link in the pathway between EGFR and nuclear transcription factors. It is interesting to note that of all the diet groups, HFB had the largest decrease in ERK-1 gene expression. Tumors from the HFB group also had a significant decrease in EGFR gene expression. It has been shown that decreased activation of EGFR leads to decreased activation of ERK-1 (Yamauchi et al., 1999). Therefore, it is possible that the decreased expression of these two genes is biologically connected, and may be a result of the HFB diet.

In summary, it appears that certain diets alter the expression of specific genes in colonic tumors compared to mucosa, based on their specific fatty acid composition.

# CHAPTER 8

# TGF-α, EGFR, CYCLOOXYGENASE AND ERK PROTEIN EXPRESSION IN COLONIC MUCOSA AND TUMORS AS AFFECTED BY THE DIETARY LIPID COMPOSITION

## 8.1 Introduction

Accumulating evidence from both clinical and experimental studies has suggested that the COX isozymes play an increasingly important role in colon cancer progression. This stems from the fact that COX inhibitors (such as piroxicam) are effective chemopreventive agents, and that increased protein expression of COX2 has been noted in human adenomas and tumors (Kargman et al., 1995; Sano et al., 1995). Furthermore, alterations in COX expression and activity are thought to be a potential mechanism for the tumor promoting or inhibitory effects of ω-6 and ω-3 fatty acids, respectively. The specific long-chain fatty acids EPA and DHA have been shown to inhibit COX activity and metabolism of AA to form prostaglandins (Cory et al., 1984). However, if these fatty acids and other chemical COX inhibitors may also affect the protein synthesis of COX remains unknown.

The presence of TGF-α and the ERKs has also been of recent interest in colon carcinogenesis, due to their role in the regulation of cell proliferation, differentiation and transformation (Nishida & Gotoh, 1992). The proposed relationship between TGF-α and its receptor, COX and ERK in cancer development has been discussed previously (Chapter 2). However, the

expression of these proteins has never been assessed in conjunction with each other in either colonic mucosa or tumors. Furthermore, how the expression of TGF-α and the ERK isoforms may be altered according to the tumor-promoting ability of specific dietary lipids has yet to be determined.

In the present study, it was of interest to compare the protein expression for TGF-α, COX1, COX2, ERK-1 and ERK-2 in both colonic mucosa and tumors from animals fed diets varying in fatty acid composition.

## 8.2 Materials and Methods

*Animals, Diets, Carcinogen, Study Protocol*

Animals used were those described in Chapter 6. Specific details pertaining to the diet composition and study protocol are described in Chapter 6, materials and methods.

*Preparation of Samples for Western Blotting*

Fresh, thawed mucosal scrapings and tumors (n=4-5/group) were placed in 2 ml of ice-cold RIPA buffer (Appendix E), homogenized with a polytron and centrifuged at 15,000 X *g* for 20 minutes. The supernatant was drawn, separated into numerous aliquots (50-100 µl each) and stored at -80°C until further analysis.

*Protein Concentration*

Protein concentration for all samples was determined as described in Chapter 3.

*Detection of Proteins by Western Blotting*

The general western blotting procedures used are detailed in Chapter 3. Recipes for polyacrylamide gels and buffers used for immunoblotting are described in Appendix D. The specific conditions used for each antibody are described in Table 8.1. To ensure that the detection of immuno-complexes by ECL was linear, initial trials were conducted for tumor and mucosa samples utilizing increasing amounts of protein (5 – 100 μg) for all antibodies. The response was linear between 30 and 60 μg of protein, with the exception of ERK-1 and –2.

*Immunohistochemical Detection of EGFR, COX-1 and COX-2*

The general protocol used for immunohistochemisty is described in Chapter 3. Sections of colonic mucosa and tumors were fixed in either ethanol or paraformaldehyde, depending on the specificity of the antibody. All antibodies were diluted with fresh PBS. The antibodies and dilutions used were: EGFR (cat#sc-003, Santa Cruz Biotechnology, Santa Cruz, CA), 1:50; COX1 (cat#160110, Cayman Chemical Co., Ann Arbor, MI), 1:200; COX2 (cat#160106, Cayman Chemical Co.), 1:200. All antibodies were incubated overnight at 4°C in a humid chamber.

*Statistical Analysis*

Statistical analysis of TGF-α, COX1, COX2, ERK-1 and ERK-2 expression was carried out using analysis of variance (ANOVA) in conjunction with Duncan's Multiple Range Test. A $P$ value of $\leq 0.05$ was considered significant.

**Table 8.1 Experimental Conditions for the Detection of Proteins by Western Blotting**

| Protein | Molecular Weight (kDa) | Amount Loaded (μg)[1] | Gel % | Antibody Dilution | Positive Control |
|---|---|---|---|---|---|
| ERK-1/2[2] | 44/42 | 15 | 12 | 1:2000 | NIH-3T3 cell lysate[2] |
| COX1[3] | 70 | 50 | 10 | 1:1000 | COX1 Standard[3] |
| COX2[4] | 72 | 50 | 10 | 1:2000 | COX2 Standard[4] |
| TGF-α[5] | 5 | 50 | 15 | 1:100 | TGF-α Standard[5] |

1. Represents the amount of protein loaded for both mucosa and tumor samples
2. Cat#06-182, Upstate Biotechnology, Lake Placid, NY
3. Cat#160110 and 360100 (control), Cayman Chemical Co., Ann Arbor, MI.
4. Cat#160106 and 360120 (control), Cayman Chemical Co., Ann Arbor, MI.
5. Cat#sc-36, Santa Cruz Biotechnology, CA, USA; Cat#PF008 (control), Oncogene, Cambridge, MA

## 8.3 Results

*Expression of TGF-α*

The main TGF-α isoforms found in colonic mucosa and tumors were 20 kDa and 61kDa, respectively. The highest tumor expression was in the HFF group (1092.3±249.5 pixels, $P<0.05$) followed by HFP, HFB and HFC tumors had the lowest (621.3±461.0 pixels). TGF-α expression in colonic mucosa was very similar among the groups, and in the decreasing order of HFC>HFP>HFF>HFB (range 42.2 to 34.0 pixels). There were no significant differences. Tumors had a much higher expression of TGF-α compared to all mucosa samples, irrespective of diet treatment (Figure 8.1). The largest increase in protein density going from mucosa to tumor was found in the HFF and HFP groups, while the smallest increase was seen in the HFC group.

*Expression of COX1*

The highest expression of COX1 was in colonic mucosa from the HFC group (1290.6±191.4 pixels, $P\leq0.05$). COX1 tumor expression was in decreasing order of HFB>HFP>HFC>HFF with a range of 771.3 to 208.3 pixels. It is interesting to the note that the HFC and HFF samples had a higher mucosa COX1 expression compared to all tumors, irrespective of diet treatment (Figure 8.2). The HFF and HFC groups also had a significantly lower expression of COX1 in tumors compared to mucosa. Alternatively, the HFB and HFP groups both had an increased COX1 expression when comparing mucosa and tumor samples.

Figure 8.1 Density of TGF-α protein expression in colonic mucosa and tumor fractions from F344 rats fed HFB, HFF, HFP or HFC diets for 16 weeks. Immunoreactive bands were observed at 21 kDa in colonic mucosa and 60 kDa in colonic tumors. Values are mean ± SD (bars) and are expressed in pixels, representing the area of the visualized protein bands. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).

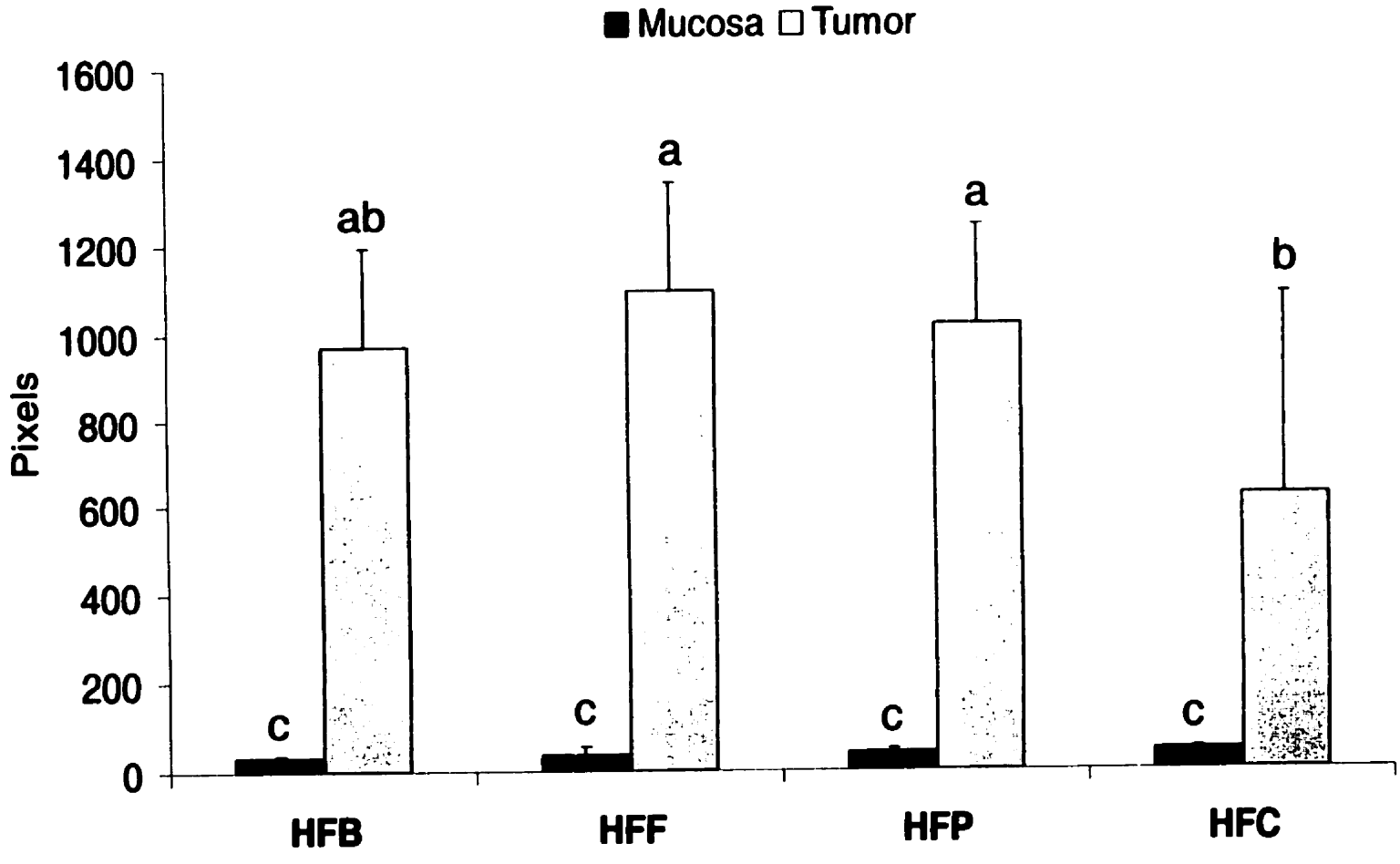
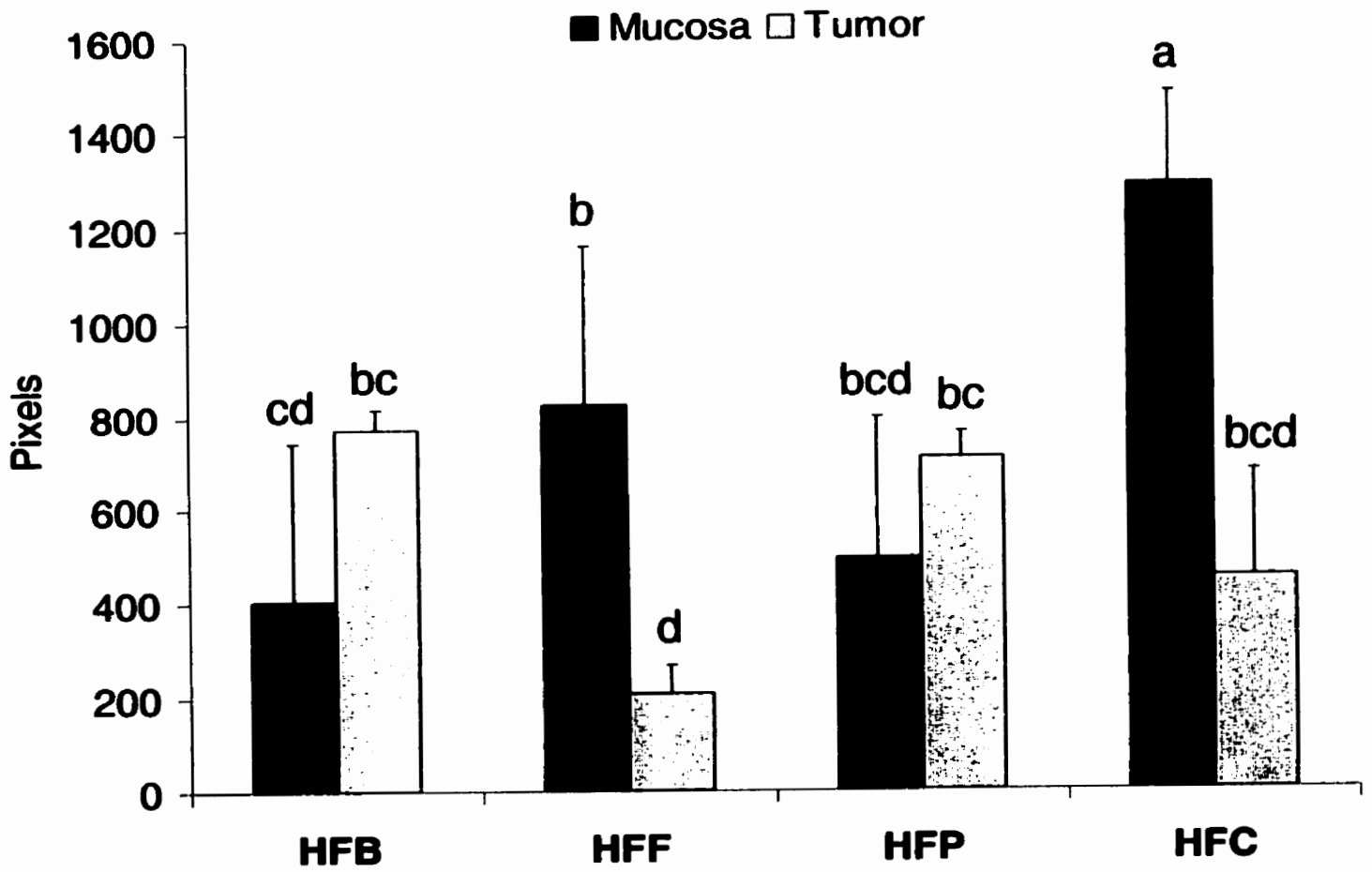

■ Mucosa □ Tumor
Pixels
1600
1400
1200
1000
800
600
400
200
0
ab
a
a
b
c
c
c
c
HFB
HFF
HFP
HFC

Figure 8.2 Density of COX1 protein expression in colonic mucosa and tumor fractions from rats fed HFB, HFF, HFP or HFC diets for 16 weeks. Values are mean ± SD (bars) and are expressed in pixels, representing the area of the visualized protein bands. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).


Mucosa
Tumor
Pixels
1600
1400
1200
1000
800
600
400
200
0
cd
bc
b
d
bcd
bc
a
bcd
HFB
HFF
HFP
HFC

*Expression of COX2*

A western blot of the expression of COX2 in colonic tumors is presented in Figure 8.3. The highest overall expression of COX2 was observed in HFF tumors (2402.5±252.7 pixels, $P \leq 0.05$). Tumors from the HFC and HFB groups had similar COX2 expression, with HFP tumors having the lowest expression (1084.0±308.9 pixels, Figure 8.4). All colonic mucosa samples had a lower expression of COX2 compared to tumors, irrespective of the diet treatment (Figure 8.4).

*Expression of ERK-1*

The detection of immunoreactive ERK-1 in colonic tumors is described in Figure 8.3. All tumor samples had a higher expression of ERK-1 compared to mucosa, with HFB tumors having the highest overall expression (693.3±56.3 pixels, $P \leq 0.05$). Tumor expression for the remaining groups was similar. ERK-1 expression in colonic mucosa was in the decreasing order of HFP>HFF>HFB>HFC (Figure 8.5). All groups had significant increases overall in ERK-1 when comparing mucosa and tumor samples.

*Expression of ERK-2*

The detection of immunoreactive ERK-2 in colonic tumors is described in Figure 8.3. Once again, the expression of ERK-2 was higher in all tumors compared to mucosa, with tumors from the HFB group having the highest overall ERK-2 expression (497.8±118.1 pixels, $P \leq 0.05$). Remaining ERK-2 expression among tumors was followed by HFP, HFC and finally HFF had the lowest

Figure 8.3 Expression of COX2 (A) and ERK-1 (B) in colonic tumors from rats fed HFB, HFC, HFF and HFP diets for 16 weeks. Protein bands were detected by enhanced chemiluminescence and developed in Kodak X-OMATAR film. Positive controls were a COX2 electrophoresis standard (Cayman Chemical Co, Ann Arbor, MI) and NIH-3T3 cell lysate (UBI, Lake Placid, NY).

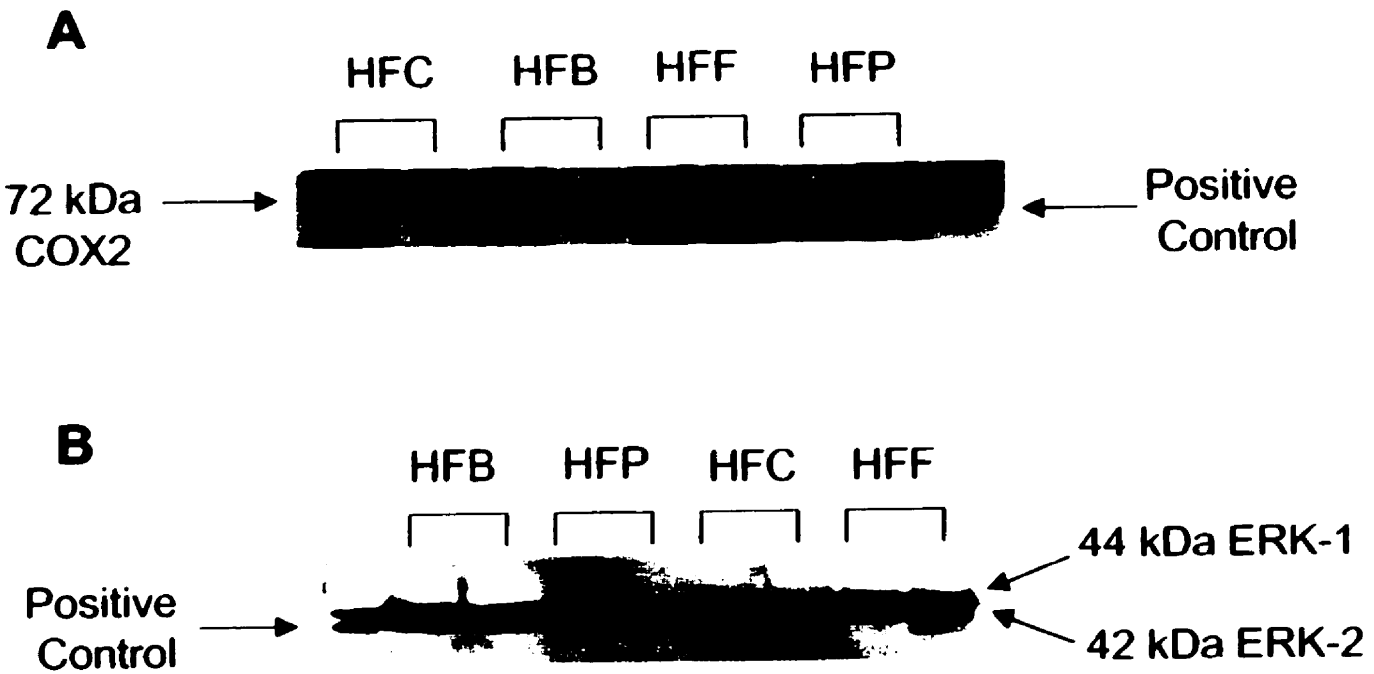

A
HFC
HFB
HFF
HFP
72 kDa
COX2
Positive
Control
B
HFB
HFP
HFC
HFF
44 kDa ERK-1
Positive
Control
42 kDa ERK-2

Figure 8.4 Density of COX2 protein expression in colonic muocsa and tumors fractions from rats fed HFB, HFF, HFP or HFC diets for 16 weeks. Values are mean ± SD (bars) and are expressed in pixels, representing the area of the visualized protein bands. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).

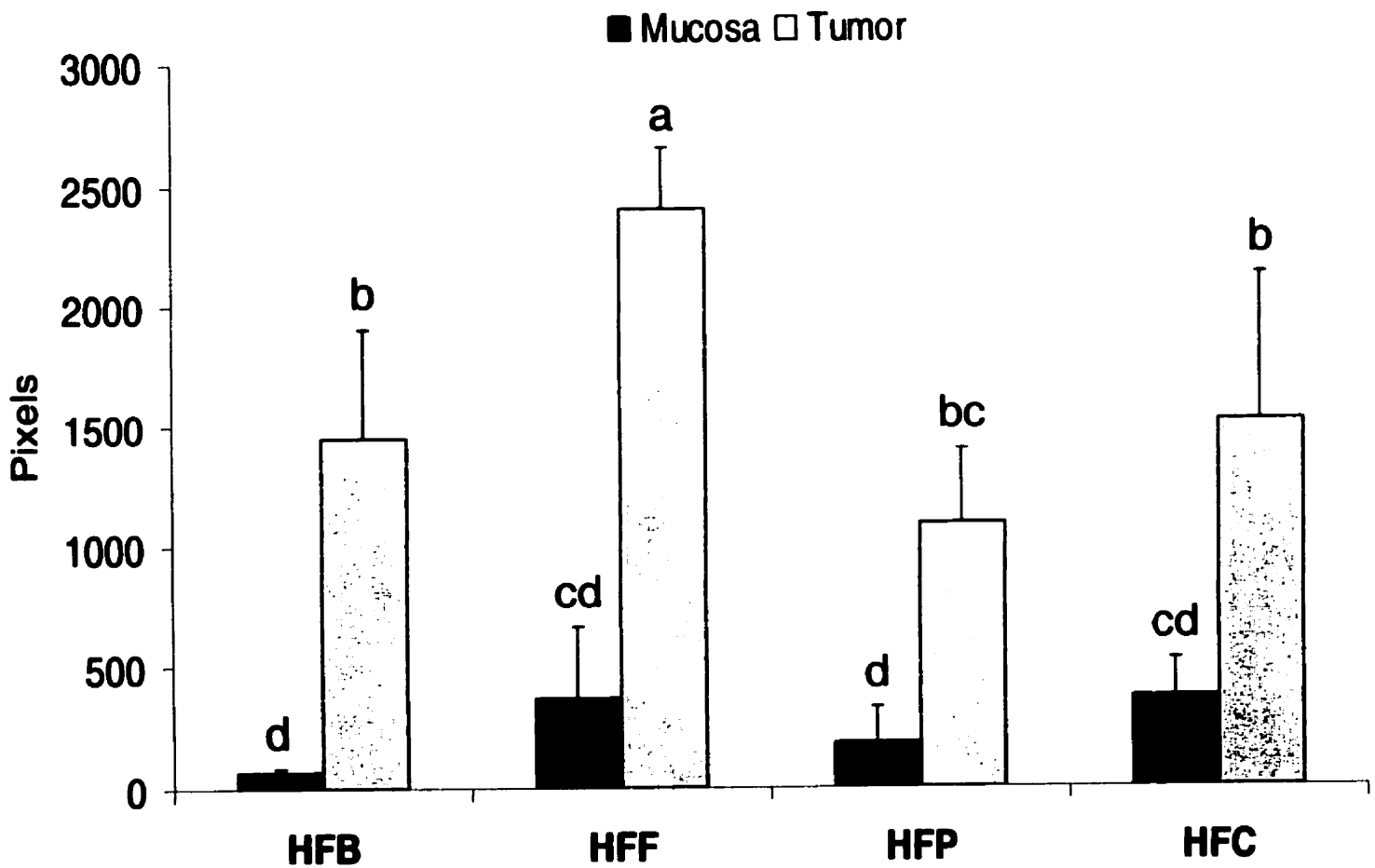
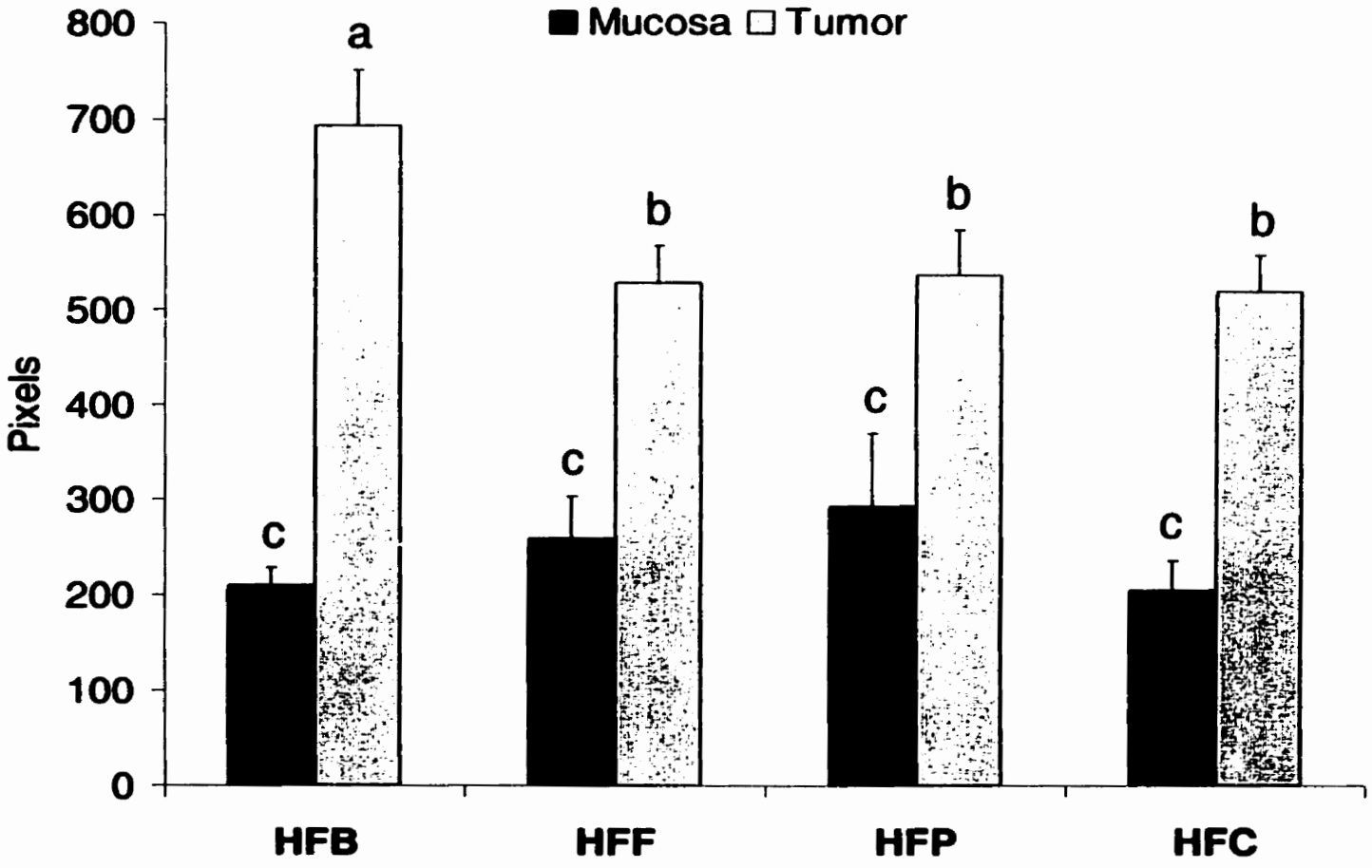

■ Mucosa □ Tumor
Pixels
3000
2500
2000
1500
1000
500
0
d
b
cd
a
d
bc
cd
b
HFB
HFF
HFP
HFC

Figure 8.5 Density of ERK-1 protein expression in colonic mucosa and tumor fractions from rats fed HFB, HFF, HFP or HFC diets for 16 weeks. Values are mean ± SD (bars) and are expressed in pixels, representing the area of the visualized protein bands. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).


Mucosa
Tumor
Pixels
800
700
600
500
400
300
200
100
0
a
b
b
b
c
c
c
c
HFB
HFF
HFP
HFC

expression (247.3±101.9 pixels). Mucosal expression of ERK-2 was almost equivalent between HFF and HFP (132.6±68.7 and 129.0±9.9 pixels, respectively), followed by the HFC group. Mucosa from the HFB group had the lowest ERK-2 expression at 78.3±57.3 pixels, as described in Figure 8.6. Although all groups had higher expression of ERK-2 in tumors compared to muocsa, only the HFB and HFP groups had significant increases ($P \leq 0.05$).

*Immunohistochemical detection of EGFR, COX1 and COX2*

EGFR expression in carcinogen-treated colonic mucosa was mainly in the upper portion of the crypt (Figure 8.7A), although some nuclear staining was present. In colonic tumors, there was an increase in nuclear staining for EGFR compared to mucosa. The nuclei also appeared to be more scattered throughout the tumor section (Figure 8.7B). Interestingly, there was a large amount of lymphocytic infiltration in the tumor section that was positively stained for EGFR. Immunoperoxidase staining for COX2 was increased in mucosa, ACF and tumor sections compared to COX1. This finding is demonstrated in Figure 8.8, where the identical section of an ACF exhibited severe dysplasia is stained for COX1 (A) and COX2 (B).

## 8.4 Discussion

The main objective of the present study was to investigate the expression of specific proteins reported to be involved in colon carcinogenesis (TGF-α, COX1, COX2, ERK-1 and ERK-2) as affected by specific dietary lipids. A comparison of the protein expression between carcinogen-treated colonic mucosa and tumors from each diet group provides insight as to changes in

Figure 8.6 Density of ERK-2 protein expression in colonic mucosa and tumor fractions from rats fed HFB, HFF, HFP or HFC diets for 16 weeks. Values are mean ± SD (bars) and are expressed in pixels, representing the area of the visualized protein bands. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).

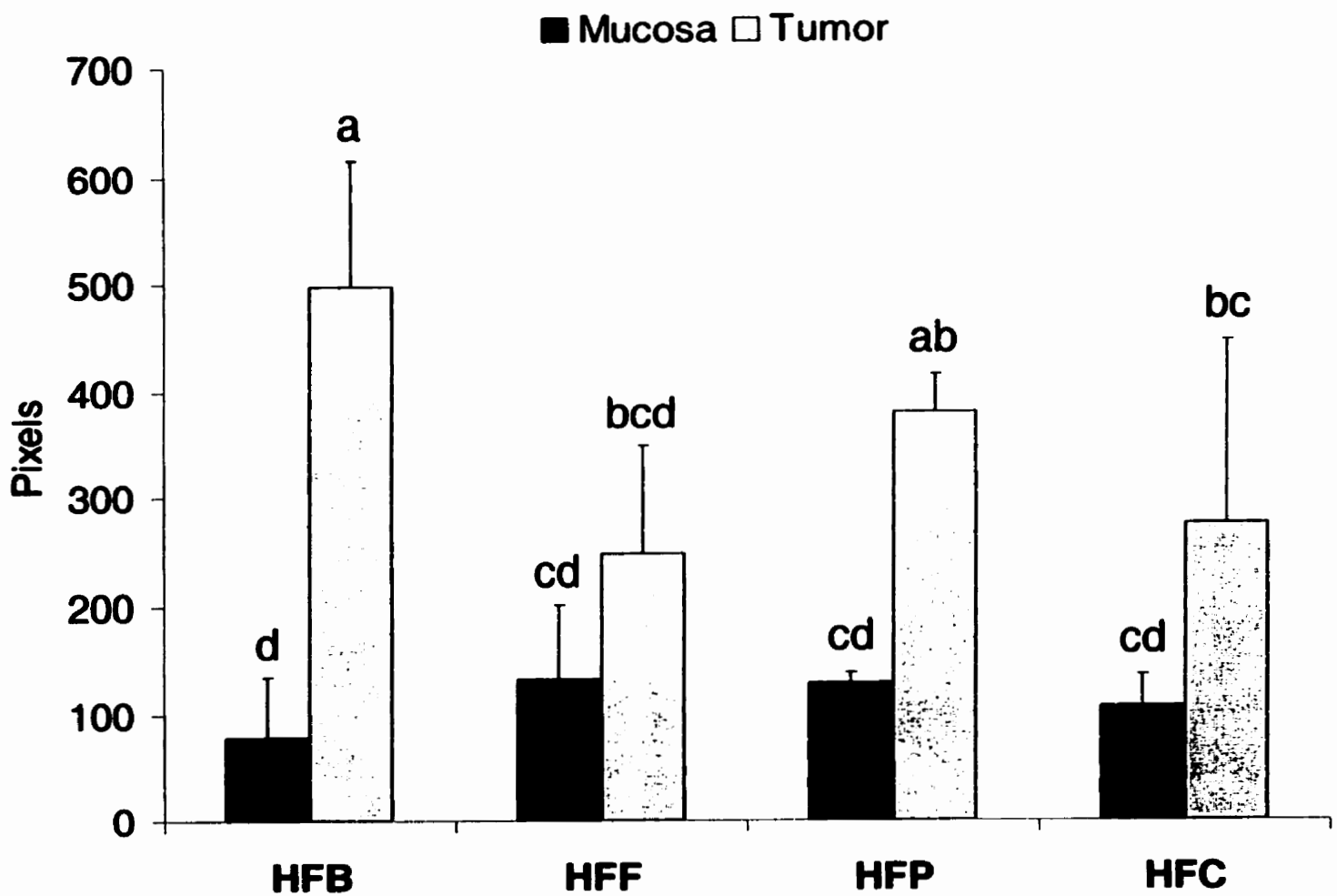

Mucosa
Tumor
Pixels
700
600
500
400
300
200
100
0
a
d
bcd
cd
ab
cd
bc
cd
HFB
HFF
HFP
HFC

Figure 8.7 Immunohistochemical staining to reveal immunoreactive EGFR in normal crypts and tumors. Note the presence of EGFR (X200) staining mainly in the upper portion of the normal crypts (A). Note the presence of strong immunoreactivity in a tumor (X200), with the presence of increased nuclear and lymphocytic infiltration (B).

Figure 8.8 Immunoperoxidase staining to reveal immunoreactive COX1 (A) and COX2 (B) in ACF and normal crypts (X200). Note the high level of dysplasia throughout the aberrant focus. Note the increase in intensity for COX2 staining in both normal mucosa and ACF compared to COX1.

specific proteins that may be critical to tumor formation and progression. Moreover, assessment of these target proteins at the same time point may demonstrate if a functional relationship exists among these signaling elements, as has been previously suggested. The main findings of the present study are as follows: 1) The HFC diet caused a significant increase in COX1 in colonic mucosa, but was significantly decreased in tumors; 2) Tumors from the HFF diet had the highest COX2 expression; 3) Tumors from the HFB diet had the highest expression of both ERK-1 and ERK-2.

Many recent studies have focused on the expression of COX in colonic tumors. As discussed previously, although the two isoforms are have been shown to be homologous in structure and ability to synthesize prostaglandins, they are thought to differ in terms of biological function (Sakamoto, 1998). COX1 has been shown to be induced slowly in crypt cells in response to inflammation or stress, whereas, COX2 increases much more rapidly. Further evidence now suggests that COX1 is critical to crypt cell survival as it maintains basal levels of prostaglandins, compared to COX2 that is more likely to a play a role in initiating repair after a cellular insult (Sakamoto, 1998). Studies in both humans and experimental animals have reported increased COX2 expression in colonic tumors compared to mucosa, while COX1 expression has generally been similar between the two tissues (Sano et al., 1995). Therefore, the results of the present study demonstrating dramatically increased levels of COX1 in colonic mucosa compared to tumors in the HFC, and also in the HFF groups were unexpected. Furthermore, the increased expression of COX2 protein in HFF tumors compared

to the other diet groups was also a surprise. However, it should be noted that the expression of COX2 was higher than that of COX1 in tumors from all diet groups, which is in accordance with the findings of others (Kargman et al., 1996; Sano et al., 1995). To the author's knowledge, only one study has previously examined colonic mucosa and tumor COX1 and COX2 expression from animals fed diets high in corn oil and fish oil (Singh et al., 1997). This study reported a decrease in both COX1 and COX2 expression in tumors from the fish oil diet compared to the corn oil diet. Expression of COX1 and COX2 in colonic mucosa was also lower compared to that in tumors from animals fed fish oil (Singh et al., 1997).

Assessment of the gene expression for COX1 (Chapter 7, Figure 7.3) revealed a slight increase in HFB tumors compared to mucosa, with the HFF, HFC and HFP having no change in expression. Immunodetection of COX1 protein in tumors and mucosa in the present study did not corroborate those findings. The expression of COX1 from mucosa and tumors in the HFF and HFC groups was completely opposite of that in the HFB and HFP groups. A large increase in mucosal expression, and then decrease in tumor expression was observed in the HFF and HFC groups.

Expression of COX2 was significantly higher in colonic tumors from the HFF group compared to the other diets. Animals fed diets high in fish oil have been reported to have a lower expression of COX2 in both colonic and mammary tumors compared to those fed a high corn oil diet (Badawi et al., 1998; Hamid et al., 1999; Singh et al., 1997). Since all groups had an increase in tumor COX2 expression, it is difficult to assess the magnitude of the effect of the experimental

diets. The only group that had an increase in COX2 gene expression compared to mucosa from the previous study was the HFC group. The differences between the results for COX2 protein and gene expression could be caused by a number of factors including an increased rate of mRNA translation or a decreased rate of protein degradation. Since COX2 is highly inducible, one would not normally see high amounts of this isoform in colonic muocsa. The fact that substantial amounts of the mRNA are present in mucosa suggests that a large increase in translation may be the response elicited during tumor formation, resulting in the increased levels of protein observed in the present study.

The increase in TGF-α expression in colonic tumors in the present study supports the findings of others, and also reinforces the proposed role of TGF-α as a mitogenic growth factor (Borlinghaus et al., 1993; Messa et al., 1998; Younes et al., 1996). Furthermore, it has been reported that serum levels of TGF-α are also increased in patients with colorectal tumors, and these levels dramatically drop after tumor resection (Shim et al., 1998). It is possible that the high levels of TGF-α secreted by angiogenic tumors may contribute to the increased serum levels observed in colon cancer patients. These findings suggest the use of TGF-α as a potential biomarker for the presence of malignancy in the lower gastrointestinal tract.

Immunohistochemical evaluation of normal mucosa, ACF and tumors for EGFR, COX1 and COX2 revealed no diet-related changes among the groups. This was not surprising, as this method is often considered to be a qualitative measure, because results can vary depending on the tissue fixative, thickness of

the tissue section, antibody dilution and incubation time. To the author's knowledge, this is the first study to assess the immunoreactivity of COX1 and COX2 in ACF. Previous studies have reported a higher amount of COX2 expression in colonic tumors as compared to COX1 (DuBois et al., 1996; Gustafson-Svard et al., 1996). A similar trend was observed in the present study, as COX2 immunoreactivity was stronger than that of COX1 in both ACF and tumors. Expression of EGFR in normal colonic mucosa was primarily seen along the top portion of crypt at the luminal surface. This finding is in parallel with previous observations of TGF-α expression in the upper portion of the crypt (Bird, 1995; Tanno & Ogawa, 1994; Thorup, 1997), revealing that the growth factor is in close proximity to its receptor.

The finding that HFB tumors had a significant increase in both ERK-1 and ERK-2 compared not only with colonic muocsa, but also tumors from the other diets groups was surprising. Previous studies have demonstrated an increase in ERK protein expression and activity in tumors compared to normal surrounding mucosa in colon cancer patients (Eggstein et al., 1999; Ostrowski et al., 1998; Sebolt-Leopold et al., 1999). A recent study by Davidson and colleagues (1999) demonstrated a 2-fold increase in ERK-1 and –2 protein expression in colonic tumors compared to mucosa from rats fed diets high in either corn oil or fish oil. Similar results were seen in the present study, however, the increase for ERK-1 and –2 in HFB tumors exceeded those observed in both the HFC and HFF groups. The effect of saturated fat on the protein expression of signaling enzymes has been investigated in few studies, and seems to have taken a

backseat to the current focus on ω-6 and ω-3 fatty acids. Therefore, the findings of the present study may provide the impetus to re-evaluate our limited understanding of the cellular mechanisms behind the tumor-promoting effects of saturated fat.

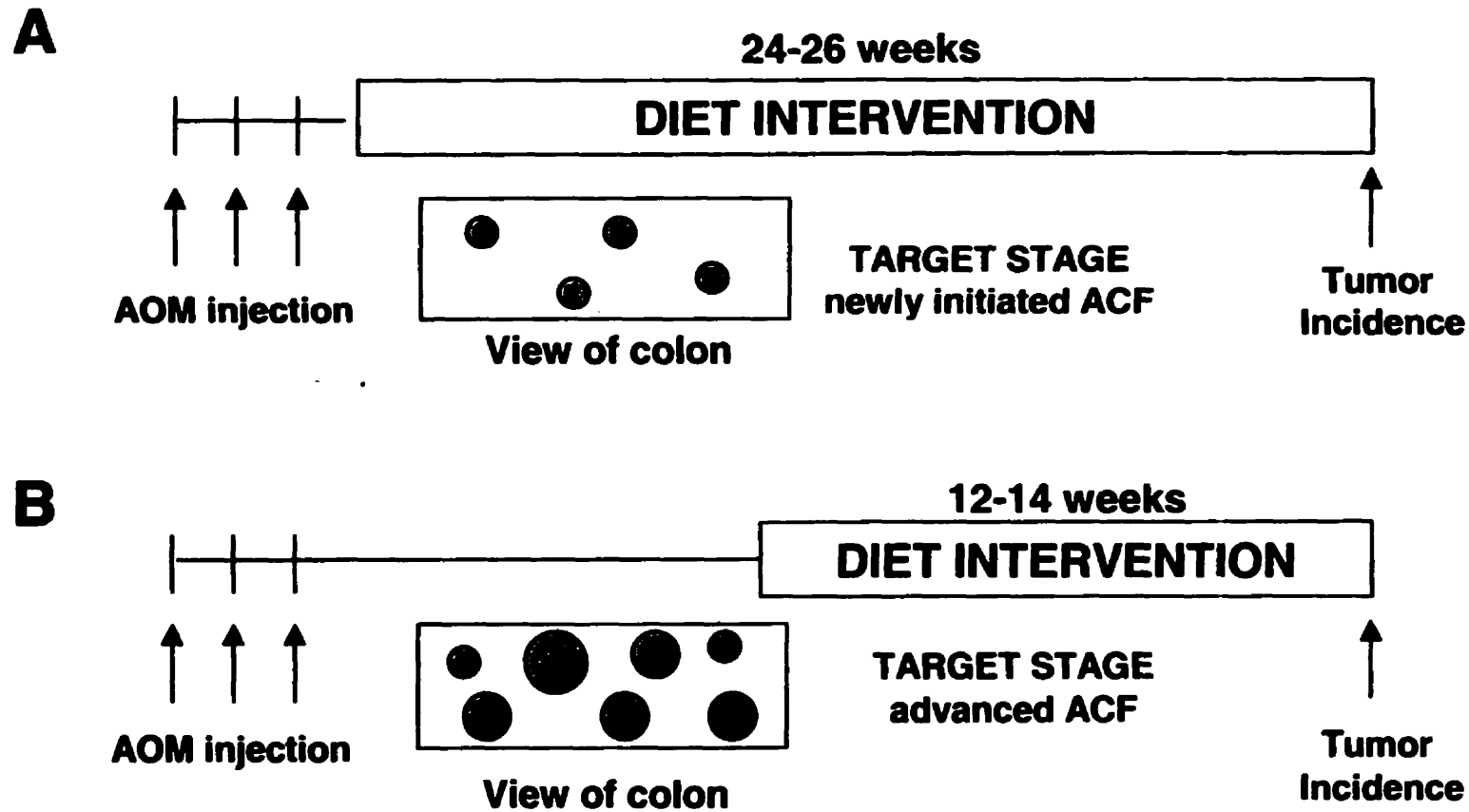
# CHAPTER 9

# 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 discussion will be to discuss the results in the context of their contributions towards further understanding the multi-step process of colon carcinogenesis.

In the past, it was common for certain chemical compounds or dietary constituents to be classified as "cancer preventive or promoting" agents solely based on their ability to increase or decrease colon tumor incidence. For the majority of these studies, diet intervention was initiated during or soon after carcinogen injection, with tumor incidence being assessed as the final end-point. The discovery of ACF and development of the ACF system has provided a simple yet effective model to study the effects of specific nutrients on the preneoplastic stages of carcinogenesis. A significant contribution of the present dissertation was the application of a delayed-intervention approach, whereby dietary intervention was delayed for at least 10 weeks after the final AOM injection (Chapters 5 & 6). Although the difference in experimental design may seem minimal, the preneoplastic stages targeted by each approach are significantly different. In the first approach, the experimental diet is affecting the growth of primal ACF that have newly appeared, or those that have not yet surfaced (Figure 9.1A). Furthermore, the experimental animals are only weanlings and still rapidly growing.

Figure 9.1 A schematic representation of the experimental protocol commonly used in diet intervention studies. The first protocol involves intervention by a diet soon after carcinogen-treatment, and therefore targets newly appearing preneoplastic lesions (A). The protocol employed in the present dissertation involves delaying diet intervention several weeks after carcinogen-injection. This enables the effect of the experimental diet to be assessed on a spectrum of preneoplastic lesions of various growth dimensions (B).


A
24-26 weeks
DIET INTERVENTION
AOM injection
View of colon
TARGET STAGE
newly initiated ACF
Tumor
Incidence
B
12-14 weeks
DIET INTERVENTION
AOM injection
View of colon
TARGET STAGE
advanced ACF
Tumor
Incidence

In the present approach, delaying diet intervention by several weeks allows primal lesions to progress to more advanced stages, as well as enables new primal populations of ACF to appear. Therefore, the effects of the experimental diet can be evaluated on lesions of various growth dimensions (Figure 9.1B). In addition, the animals have reached maturity at the time of diet intervention, and are more stable in terms of weight and size. This approach enables the assessment of all carcinogenic stages at a single time point, thereby providing further insight into which stage may be most responsive to the growth effects of the experimental diet. It is known that individuals at high risk for colonic cancer already harbor precancerous lesions of different growth dimensions. Taking this into consideration, one can firmly conclude that the experimental approach taken in the present dissertation most closely reflects the pathogenic state of humans at high risk for colon cancer. The practical applications of the delayed-intervention approach are many, as it provides a standardized method to assess the chemopreventive potential of any nutrient or food component. This may eventually lead to the identification and application of specific cancer preventive agents capable of targeting specific stages of colon cancer development.

Once thought to be simply a main source of energy, recent evidence has revealed the influence of dietary lipids on cell growth and differentiation, plasma membrane composition, signal-transduction pathways, and nuclear gene expression (Jump et al., 1999; Jump et al., 1997; LaFave et al., 1994; Reddy, 1994). Investigation of the role of specific dietary lipids in colon carcinogenesis

from both epidemiological and experimental perspectives has grown rapidly in the last decade. Several animal studies have supported the inhibitory effects of fish oil, based in its reported ability to reduce colonic tumor incidence by up-to 50% compared to a high-corn-oil diet (Caygill et al., 1996; Chang et al., 1998; Reddy, 1994; Singh et al., 1997). These studies began feeding a high fish oil diet to young animals during or immediately following carcinogen injection. A similar approach was employed in a few studies that reported the inhibitory effects of EPA and DHA on the growth of ACF (Takahashi et al., 1997, Takahashi et al, 1994).

When the effect of fish oil was assessed on established ACF in present dissertation, it was found to have a very strong promoting effect on the growth of advanced preneoplastic lesions (Chapter 5). Although the final tumor incidence was lower than the HFC and HFB groups (47% vs. 65 and 57%, respectively), it was not as dramatically decreased as reported by previous studies. Furthermore, the number of adenomas in the HFF group was similar to the tumor-promoting diets containing beef tallow or corn oil. These findings demonstrate that only specific advanced lesions may be responsive to the reported "inhibitory" effects of fish oil. More specifically, fish oil may have prevented the progression of MA to form tumors, resulting in a lower tumor incidence. However, the knowledge that fish oil greatly enhanced the progression of established precancerous colonic lesions, combined with the current advocation for increased fish consumption and use of fish oil as a chemopreventive agent in high-risk individuals is troubling.

The main objective in comparing fish oil with the COX inhibitor piroxicam was to investigate if the purported inhibitory effect of fish oil was due to its influence on prostaglandin synthesis, as has been suggested. In this study, the experimental diets were fed for 16 weeks (Chapter 6), compared to 12 weeks in the previous study. Piroxicam, even in conjunction with a high corn oil diet, inhibited colonic tumor incidence more effectively than fish oil (30% vs. 57%, respectively). Furthermore, the HFP and HFF groups appear to have opposite effects on the growth of advanced ACF (Table 6.3). The HFP group decreased the number of ACF with >4 crypts/focus compared to fish oil. Whereas, the HFF group displayed a similar outcome as the previous study (Chapter 5), in terms of enhancing the growth of intermediate and advanced ACF. With the longer period of diet intervention (16 weeks) the HFF group had higher tumor incidence, total number of tumors and a much lower number of MA compared to the previous study. These findings suggest that as the duration of HFF feeding increased, a larger number of adenomas may have been able to progress and form tumors.

These findings raise important questions regarding the chemopreventive validity of fish oil. Firstly, most studies reporting the effect of fish oil in reducing colonic tumor incidence were initiated on young animals harboring a small number of primal ACF. It is known that immature precancerous lesions respond with greater sensitivity to chemopreventive agents compared to advanced, more established lesions (Bird, 1995). Similar reasoning applies to the effects of chemopreventive agents in weanling compared to adult experimental animals. The impact of the age of the experimental animals as an experimental variable is

often overlooked. From this, it could be concluded that the reports of fish oil decreasing colonic tumor incidence are attributed to its specific inhibitory effects on the growth of newly-initiated precancerous lesions in young experimental animals. Moreover, that advocation of the chemopreventive properties of fish oil in colon carcinogenesis based on these studies are potentially misleading.

Recent evidence also suggests that the long-term effects of fish oil supplementation may be unfavorable. It was demonstrated that a high fish oil diet increased colon carcinoma metastasis to the liver in rats by 10-fold compared to a low fat diet (Griffini et al., 1998). Furthermore, to the author's knowledge, the only clinical study investigating the effects of fish oil in patients harboring colonic adenomas recently reported an increase in endometrial, lung and colon cancer incidence after 2 years of supplementation (Akedo et al., 1998). Considering the results of the above studies, plus those of the present dissertation, it is evident that the scientific community needs to re-evaluate the value of fish oil as a long-term chemopreventive approach for adults with a predisposed risk for colon cancer. Time is also of the essence in this situation, as fish (menhaden) oil supplements are readily available in the marketplace, with the content and purity remaining largely unregulated.

Another main objective of this dissertation was to explore the effect of dietary lipids on the proposed signaling relationship between TGF-$\alpha$, its receptor EGFR, and the COX enzymes in colon cancer development (Coffey et al., 1997). The assessment of the ERKs was also included, as they are suggested to act as a link between EGFR, COX and nuclear transcription factors (Graves et al.,

1995). Quantification of the expression of these target genes in carcinogen-treated colonic mucosa and tumors failed to clearly demonstrate a positive association among these signaling elements for any specific diet (Chapter 7). However, it was interesting to note that individual genes appear to be up-regulated by specific dietary lipids.

The up-regulation of COX2 in HFC tumors was in accordance with present theories regarding the tumor-promoting effect of linoleic acid through increased prostaglandin production (Rao et al., 1996). The addition of piroxicam to the HFC diet appeared to prevent this increase, suggesting that this compound may affect the both enzyme activity and gene expression of the COX isoforms. Furthermore, increased expression of EGFR in colonic tumors by piroxicam is a very significant finding, considering the inhibitory potency of piroxicam on tumor development, and the current use of EGFR as a marker of malignancy potential. The HFB diet increased the COX1 and TGF-$\alpha$ expression in tumors. The expression of tumor ERK-1 expression, which has rarely been assessed at the genetic level, decreased in all groups.

Equally surprising was the fact that in the HFF group, the expression of all primers (with the exception of ERK-1) was similar between colonic mucosa and tumor samples. This suggests that the genetic effects of HFF on the growth of ACF and tumors may not involve the proteins assessed in the present investigation. Alternatively, it may also indicate that the current expression of these genes in colonic mucosa was appropriate for the growth of specific advanced ACF to form tumors, and did not require any alteration. This may

provide preneoplastic lesions from fish oil fed animals a natural advantage compared to those in animals fed the other diets, as less genotypic change is required for growth progression. Interestingly, the protein expression of these cellular components did not always corroborate their expression at the genetic level (Chapter 8). The differences between colonic mucosa and tumor expression for all proteins were much greater in all diet groups than were observed at the genetic level. The exception was the mucosal protein expression of COX1 in the HFC and HFF groups, which were significantly higher than their respective tumors. Both ERK-1 and ERK-2 protein expression were significantly increased in HFB tumors. This observation is very intriguing, as the mechanisms behind the tumor promoting effects of saturated fats have been relatively ignored compared to those of other PUFAs. Although the final tumor incidence between beef tallow and corn oil is very similar, the findings of the present studies demonstrate that the biological, cellular and molecular events underlying their positive growth effects are distinctly different.

The present dissertation was the first to not only simultaneously examine these proteins in the development of colon carcinogenesis, but also to investigate the effect of dietary fatty acid composition on their proposed inter-relationship. The attempt to relate an association between TGF-α, EGFR, and COX in tumor growth was based on previous findings derived from *in vitro* studies in established colon cancer cell lines. Mucosa and tumor fractions contain a variety of cell types, compared to *in vitro* systems that examine the responses of a single cell type under sterile conditions. The additional challenge involved when

working with animal models is that one must consider other systemic and biological factors that may influence the final results. The age of the experimental animals must also be taken into consideration, as it is known that the levels of many endocrine factors change with age. The protein levels of mucosal TGF-α in the preliminary study from young animals was much greater than those found in the mucosa from older animals (Chapter 6). It has been shown that TGF-α levels are decreased in the adult compared to weanling rat gastrointestinal tract (Perez-Thomas et al., 1993).

The role of TGF-α as a mitogenic growth factor in all stages of colon carcinogenesis still remains unclear. This is due to the fact that immunohistochemical evaluation of TGF-α in ACF has revealed a lack of immunoreactive TGF-α in preneoplastic lesions compared to tumors and normal surrounding mucosa (Bird, 1995; Thorup, 1997). Immunohistochemical evidence of this finding is provided is Figure 9.2. Previous studies have demonstrated that ACF do produce mRNA transcripts for TGF-α (Bird & Good). This suggests that a lack of immunoreactive TGF-α in ACF may be due a post-transcriptional modification of the mRNA. Alternatively, it is possible that TGF-α protein is synthesized, but rapidly degraded or secreted from these lesions, or perhaps

Figure 9.2 Immunoperoxidase staining to reveal immunoreactive TGF-α in ACF, normal crypts and tumors (X200). Note a focus of aberrant crypts lacks TGF-α immunoreactivity, whereas, normal epithelium exhibits intense staining (A). Note the presence of strong TGF-α immunoreactivity in a tumor compared (B), compared to the normal crypts.

ACF rely on TGF-α produced from the surrounding normal crypts. Furthermore, the finding that carcinogen-treated mucosa and tumors expressed immunoreactive protein bands of different molecular weight TGF-α was unexpected (21 kDa and 60 kDa, respectively, Chapter 8), and suggests that the cellular processing of pro-TGF-α may be regulated differently in normal vs. neoplastic tissues. It is unknown if the biological activities of these proteins are also different, and if they act through an autocrine, paracrine or juxtacrine mechanism(s). The questions raised by these findings add support to the contention that the production and role of TGF-α in normal, preneoplastic, and neoplastic tissues requires further investigation.

The findings of the present studies have demonstrated that certain diets can affect the growth of specific populations of ACF more than others (HFF vs. HFP). This encourages a form of natural selection among lesions, as not all ACF will progress to the next stage of development. This theory is further complicated by the fact that certain lesions acquire growth autonomy at an early stage allowing them to grow very rapidly, and are therefore unresponsive to the effects of diet intervention (Bird, 1995). This may be explain why early intervention with a diet high in fish oil appears to be more effective in preventing the growth of ACF compared to delayed intervention. It has been demonstrated that the growth of cancer cells can be inhibited by increased proliferation in the surrounding population of normal cells (van der Wal et al., 1997; Magnuson et al., 1994). The increased LI in normal colonic mucosa from weanling animals fed the HFF diet (Chapter 4) suggests that increased proliferation may play a role in

inhibiting the development of primal ACF soon after carcinogen injection, as demonstrated in previous studies (Reddy, 1994; Singh et al., 1997; Takahashi et al., 1994).

This dissertation has soundly demonstrated that the phenotypic and genotypic features of colonic tumors differ markedly from those of carcinogen-treated mucosa. When feeding diets of varied lipid composition for several weeks, one must take into consideration that a certain level of cellular adaptation must occur during that time. This is in direct contrast to cell culture systems, in which incubation with specific fatty acids is likely to produce a more immediate and distinctive cellular response. As a matter of fact, one can argue that cellular and molecular changes occurring after several weeks of chronic intervention maybe more relevant to the carcinogenic process than those observed in cell culture systems. The main theories of cellular transcription and translation lead one to assume that up-regulated gene expression is associated with an increase in protein synthesis. Although this was the case for select primers (COX2 in HFC tumors), observed trends in gene expression for a specific diet group were not always consistent with protein expression.

The proteins examined in the present dissertation are members of a diverse cellular signaling network. Other biological variables can influence their activity and expression through "cross-talk" between signaling pathways. In addition, it is known that proteins are able to regulate their own rate of gene expression and translation by feed-back inhibition (Rosenwald, 1996). Therefore, inconsistencies in expression at the nuclear and cytoplasmic levels

within a diet group may be the result of changes in the cellular environment. The large increase in protein expression in tumors compared to mucosa without a concomitant increase in gene expression may also depend on the stability of the mRNA, and/or rate of translation. A recent finding indicates a decreased rate of mRNA turnover in tumors compared to normal tissues due to a lower amount of RNA degradation enzymes (Rosenwald, 1996). These data illustrate the importance of routinely examining the various levels of cellular regulation, as the assessment of only protein or gene expression may draw false conclusions.

In a final summary, the findings of the present dissertation support the hypothesis that specific dietary lipids affect the growth of specific populations and stages of precancerous colonic lesions. This observation provides strong support for the contention that biological heterogeneity exists among ACF and tumors depending on the dietary milieu. Further examination revealed that dietary lipids cause selective alteration of specific cellular and molecular determinants depending on their fatty acid composition. The unique experimental approach employed in these studies substantiates the use and validity of the ACF system as a tool to study the effects of specific nutrients on all stages of colon carcinogenesis. Future technological developments may allow examination of the genotypic and phenotypic dimensions of specific populations of ACF at various stages of development. This information would greatly enhance our understanding of precancerous colonic stages, and eventually lead to the formation of more effective cancer prevention strategies for high-risk individuals.

# CHAPTER 10

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# CHAPTER 11

# APPENDIX A

# MODULATION OF EGFR-TK ACTIVITY AND TGF-α EXPRESSION BY DIETARY LIPIDS IN CARCINOGEN-TREATED COLONIC MUCOSA IN MALE F344 RATS

## A.1 Introduction

We previously demonstrated that activation of EGFR-TK by TGF-α was influenced by the dietary fatty acid composition in normal rat colonic mucosa (Chapter 4). In addition, quantification of TGF-α protein expression by immunoblotting revealed a trend similar to the EGFR-TK activity. After feeding experimental diets for six weeks, it was found that found that diets high in corn oil and fish oil had higher EGFR-TK activity, and significantly higher expression of TGF-α compared to a high beef tallow, or low-fat corn oil diet. One mechanism by which dietary lipids may affect EGFR activation is by altering the plasma membrane fatty acid composition to reflect their own lipid characteristics.

Although this study was the first to investigate the effects of dietary constituents on EGFR activity, previous studies have shown that carcinogen administration and age of the experimental animals affect colonic EGFR-TK activation. Malecka-Panas and colleagues (1996) reported an increase in TGF-α-induced colonic EGFR-TK activity in older rats (24 months) compared to younger rats (4 months). There was also an increase in phosphorylation of other signaling proteins associated with EGFR activation, such as ERK-1 and ERK-2,

in colonic mucosa from older animals (Malecka-Panas et al., 1996). This suggests an increased responsiveness to TGF-α in the colon with aging. Furthermore, as cancer development is considered to be a disease associated with aging, the colon may become less resistant to the mitogenic effects of TGF-α as time progresses.

Carcinogen treatment of colon mucosa has been associated with the induction of certain critical signaling enzymes such as ornithine decarboxylase, tyrosine kinase and PKC (Lafave et al., 1994; Reddy, 1994). Relan and colleagues (1995) observed a large increase in TGF-α concentration and EGFR-TK activity seven days after AOM exposure in isolated rat colonocytes. It is unclear whether increased EGFR-TK after AOM injection is merely a systemic response, or if these levels are sustained in the months following carcinogen exposure.

The previous study (Chapter 5) described the tumor modulating effects of specific dietary lipids. Diets high in beef tallow and corn oil promote colonic tumor incidence compared to a diet high in fish oil or a low-fat diet. However, it is evident that their effects on the growth of preneoplastic lesions are distinctly different. Based on the previously discussed evidence, it is possible to suggest that the mechanisms involved in the growth regulation of ACF by dietary lipids may be related to TGF-α mediated signaling through EGFR. Therefore, the objectives of the present investigation were to determine the effects of specific dietary lipids on TGF-α protein expression, and activation of EGFR-TK in carcinogen treated colonic mucosa harboring preneplastic lesions.

## A.2 Materials and Methods

*Animal Care and Experimental Diets*

The animals were housed and cared for according to Chapter 5. The diets were based on a semisynthetic AIN-76A standard diet as described in Chapter 4.

*Experimental Design*

The experimental protocol was the same as described in Chapter 5 (Figure 5.1). After 12 weeks of feeding the experimental diets (24 weeks after the final AOM injection), all remaining animals were terminated by $CO_2$ asphyxiation. Colons of all animals were excised, flushed with cold PBS, slit from cecum to anus and examined for tumors. Colons designated for biochemical analysis (5 colons/group) were placed flat on a cold plate and scraped with a clean microscope slide to collect mucosal scrapings only. Collected scrapings were immediately placed into cryovials and placed in liquid nitrogen. Samples were stored at -80°C for approximately 1 month until further analysis.

*Protein Analysis*

Protein content was determined as described in Chapter 3.

*Sample Preparation, EGFR Immunoprecipitation and EGFR-TK Assay*

All methods were performed as described in Chapter 3, and assessed on 5 samples from each diet group, in duplicate.

*Immunodetection of TGF-α*

The expression of TGF-α was determined in cytosolic fractions of all samples according to the conditions described in Chapter 3. Equal amounts of

protein (75 μg) were used for each sample. Purified TGF-α was used as positive control (Oncogene, Cambridge, MA).

*Statistical Analysis*

Statistical analysis of TGF-α isoform and EGFR-TK data was carried out using analysis of variance (ANOVA) in conjunction with Duncan's multiple range test. For all tests, a $P \leq 0.05$ was considered significant.

**A3. Results**

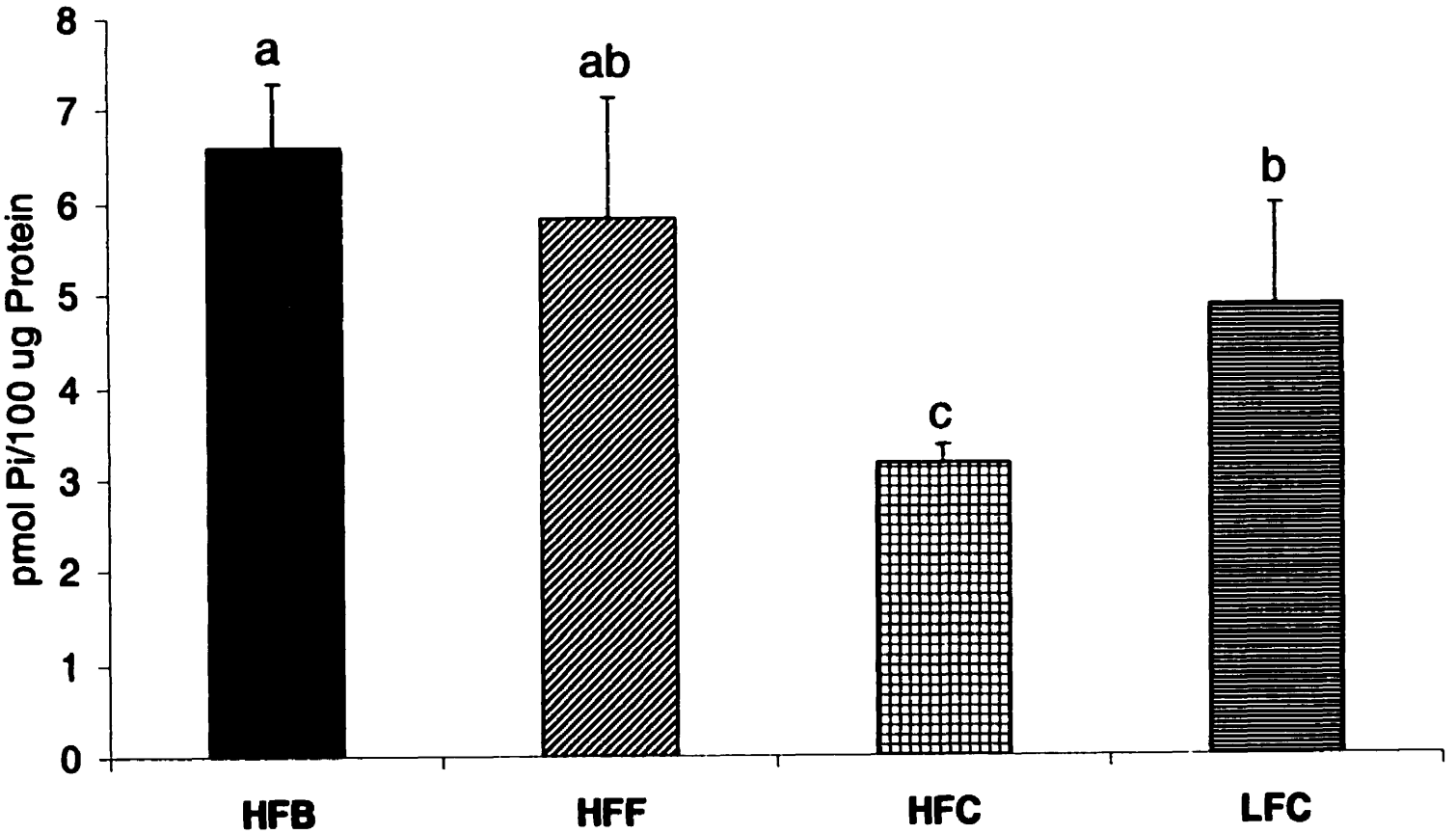
*EGFR-TK Assay*

The HFB group had the highest TGF-α stimulated EGFR-TK activity compared to all the other diet groups (6.58±0.70 pmol Pi/100 μg protein) as described in Figure A1. This was followed by the HFF, LFC and finally the HFC group with the lowest EGFR-TK activity (3.15±0.22 pmol Pi/100 μg protein).

*Detection of TGF-α by Immunoblotting*

Detection of TGF-α protein expression by western blotting revealed two distinct isoforms at 35 kDa and 15 kDa, respectively, in all diet groups. No detection of the mature 5 kDa form of TGF-α was observed in all samples. The average density of the 35 kDa protein band was significantly higher on the HFC group compared to the other high-fat diet groups (Figure A2). It was interesting to note that the LFC control group had the second highest protein expression of the 35 kDa band followed by the HFB group. Animals fed the HFF diet had the lowest band density at 35 kDa (567.7± 18.7 pixels, $P \leq 0.05$).

Figure 11.A.1. Quantification of TGF-α stimulated EGFR-TK activity in carcinogen-treated colonic mucosa from F344 rats fed HFB, HFF, LFC or HFC diets for 12 weeks. Values are mean ± SD (bars) and are expressed in pmol Pi/100 μg protein. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).


pmol Pi/100 ug Protein
8
7
6
5
4
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a
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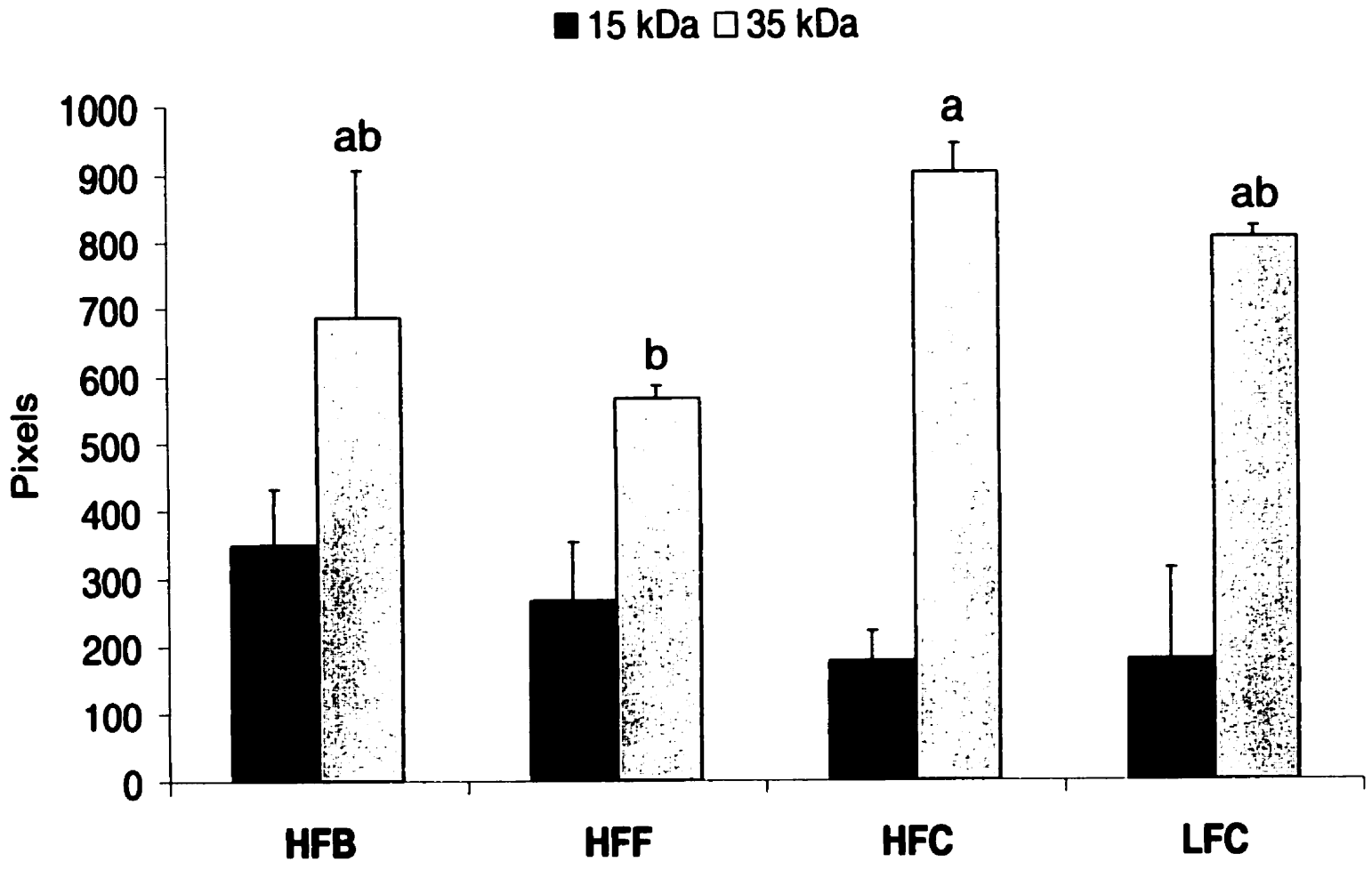
HFB
HFF
HFC
LFC

Figure 11.A.2. Density of TGF-α protein expression in carcinogen-treated colonic mucosa from F344 rats fed HFB, HFF, LFC or HFC diets for 12 weeks. Immunoreactive bands were observed at 35 kDa and 15 kDa. Values are mean ± SD (bars) and are expressed in pixels, representing the area of the visualized protein bands. Bars without common letters are significantly different ($P \leq 0.05$, Duncan's Multiple Range Test).


15 kDa
35 kDa
Pixels
1000
900
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400
300
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0
ab
b
a
ab
HFB
HFF
HFC
LFC

A contrasting trend was seen in the TGF-α isoform found at 15 kDa. The average band density was in the descending order of HFB>HFF>HFC>LFC. There were no significant differences among the diet groups. It should be noted that the band density was more intense at 35 kDa than at 15kDa for all samples (Figure A2). The HFC group had the highest total TGF-α protein expression (1078.3±131.0 pixels), followed by the HFB group. The HFF and LFC groups were very similar in terms of total TGF-α expression (745.3±178.9 and 717.0±483.0 pixels, respectively).

**A4. Discussion**

The present study was conducted to determine the ability of specific dietary lipids to modulate the protein expression of TGF-α, and its ability to stimulate the tyrosine kinase-associated with its receptor, EGFR, in carcinogen treated colonic mucosa. The specific dietary lipids used were chosen in order to investigate the potential mechanisms behind their ability to modulate the growth of preneoplastic and neoplastic colonic lesions (Chapter 5). The main findings of the present study are as follows: 1) The dietary lipids known to promote colonic tumor growth (HFB, HFC) had dramatically different effects on EGFR-TK activity; 2) Total TGF-α protein expression was similar among all diet groups; 3) The HFB diet had the highest EGFR-TK activity compared to all other experimental diet groups.

Values for EGFR-TK activity from the present study were higher for all groups compared to non-injected animals fed the same diets. It is interesting to note that when EGFR-TK was assessed in weanling animals fed an HFB diet for

only six weeks, it had the lowest activity of all diet groups. Whereas, in the present investigation using rats that were seven months old and injected with AOM, the HFB diet had a significantly higher EGFR-TK activity. In the previous study animals receiving a high beef tallow diet also had the highest tumor incidence compared to the other high fat diet groups (Chapter 5). It is not clear whether the dramatic increase in EGFR-TK observed in HFB is due to the increased age of the animals, carcinogen treatment, or an increased cellular response to TGF-$\alpha$ stimulation. It is also possible that an increased number of EGFR receptors are present on the cell surface of colons from animals fed a high beef tallow diet.

Based on that rationale, why would the high fish oil diet, shown to be non-tumor promoting, have the second highest EGFR-TK activity? An explanation could be that at the time sample collection (24 weeks after the final AOM injection), colons from animals fed the HFF diet had a significantly higher number of ACF. Mucosal scrapings contain a mix of ACF and normal epithelial cells. Therefore, an increased proportion of ACF would be present in the sample, and could contribute to the EGFR-TK activity or vice-versa. However, one could also argue against that theory, since the HFF diet had the highest EGFR-TK in non-injected mucosa, which may suggest that the presence of ACF and/or carcinogen treatment did not heavily influence the results of the present study. The HFC group did have the significantly lowest EGFR-TK activity of all diet groups, and interestingly also had the lowest number of ACF at the final termination point in the previous study. However, due to the fact that the level and activity of EGFR

in ACF is unknown, it is difficult to assess their potential contribution to total mucosal EGFR-TK.

A recent study comparing EGFR-TK activity in human colonic biopsies demonstrated a sequential increase in activity going from normal, adenomatous and cancerous mucosa (Malecka-Panas et al., 1997). These findings suggest an increase in EGFR-TK is associated with an increased risk for colon cancer. Elevated levels of EGFR have been shown to be a strong predictor of metastatic potential in colon cancer cell lines (Radinsky et al., 1995). Furthermore, newly designed compounds that specifically inhibit EGFR-TK in colon cancer cells also induce apoptotic cell death (Karnes et al., 1998), demonstrating an association between EGFR activation and neoplastic cell growth.

As mentioned previously, various isoforms of TGF-α have been detected in both normal and neoplastic tissues. Although the mature form of TGF-α was not detected in the present study, two distinct bands were observed at 35 kDa and 15 kDa. These findings complement the size of TGF-α isoforms found in non-injected mucosa at 45 kDa and 15 kDa, respectively, from animals fed the same experimental diets. Expression of the 35 kDa protein was higher in all groups compared to the expression of the smaller TGF-α isoform, although the total expression of TGF-α was similar among all the groups (Figure A2). It is interesting to note, however, that the expression of the 15 kDa isoform followed the same trend as the EGFR-TK activity (HFB>HFF>LFC>HFC). The HFC group had the highest density of the 35 kDa isoform, and yet the lowest for the 15 kDa isoform despite having a similar total TGF-α expression to the HFB group. These

findings suggest that the smaller isoform may possess higher biological activity, and ability to stimulate EGFR compared to the larger TGF-α proteins. In addition, these data demonstrate that processing of the precursor forms of TGF-α is influenced by the dietary fatty acid composition.

# APPENDIX B

## COMPOSITION OF AIN-76A VITAMIN AND MINERAL MIX

| *Vitamin Mix, AIN-76A* | g/kg |
|---|---|
| Thiamin HCl | 0.6 |
| Riboflavin | 0.6 |
| Pyridoxin 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 D3 trituration | 0.25 |
| Menadione sodium bisulfate complex | 0.15 |
| Sucrose, fine powder | 981.08 |

| *Mineral Mix, AIN-76A* | g/kg at 3.5% of diet |
|---|---|
| Calcium | 5.155 |
| Phosphorous | 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 |

# APPENDIX C

**Table C1. Fatty Acid Composition of Experimental Lipids [1,2]**

| Fatty Acid | Beef Tallow | Corn Oil | Fish Oil |
|---|---|---|---|
| 14:0 | 3.1 | ND | 10.5 |
| 16:0 | 23.0 | 10.1 | 16.1 |
| 16:1 | 2.2 | ND | 12.7 |
| 18:0 | 19.4 | 1.7 | 2.9 |
| 18:1n-9 | 38.6 | 26.5 | 9.0 |
| 18:2n-6 | 3.4 | 59.2 | 1.1 |
| 18:3n-3 | 1.0 | 0.8 | 1.0 |
| 20:5n-3 | ND | ND | 16.4 |
| 22:5n-3 | ND | ND | 3.0 |
| 22:6n-3 | ND | ND | 8.0 |

1. Expressed as percent composition, ND, non-detectable
2. Fatty acid composition assessed by gas chromatography

**Table C2. Fatty Acid Composition of Experimental Diets [1,2]**

| Fatty Acid | Beef Tallow | Corn Oil | Fish Oil |
|---|---|---|---|
| 14:0 | 2.4 | ND | 8.2 |
| 16:0 | 20.6 | 10.6 | 14.8 |
| 16:1 | 1.8 | 0.1 | 9.9 |
| 18:0 | 16.0 | 2.2 | 3.2 |
| 18:1n-9 | 34.9 | 25.5 | 12.2 |
| 18:2n-6 | 14.3 | 57.8 | 12.3 |
| 18:3n-3 | 2.6 | 2.4 | 2.3 |
| 20:5n-3 | ND | ND | 12.8 |
| 22:5n-3 | ND | ND | 2.3 |
| 22:6n-3 | ND | ND | 6.2 |

1. Expressed as percent composition, ND, non-detectable
2. Fatty acid composition assessed by gas chromatography

# APPENDIX D

## RIPA Buffer for EGFR-TK Assay

As detailed in Current Protocols in Molecular Biology, ed. Asubel et al., 1995 (volume 2)

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
5mM PMSF
10 μg/ml leupeptin
1 mM $Na_3VO_4$
5 mM NaPP
20 mM sodium phosphate
1 μl/ml aprotinin

Stored at 4°C.

# APPENDIX E

Western Blotting Buffers and Acrylamide Gel Recipes (based on the method described by Laemmli, 1970). All reagents and recipes are from Bio-Rad.

*30% Acrylamide/bis mix*

87.6 g acrylamide (29.2 g/100 ml)

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

Made to 300 ml with deionized $H_2O$ and stored at 4°C in the dark.

*10% Ammonium Persulfate*

100 mg ammonium persulfate in 1 ml deionized water.

*10% Sodium Dodecyl Sulphate (SDS)*

10 g SDS dissolved in 90 ml $H_2O$ with gentle 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 (N,N,N-tetramethylethyl-enediamine) | 0.005 ml |
| | 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 Electrode Running Buffer*

| | |
|---|---|
| Tris Base | 9 g |
| Glycine | 43.2 g |
| SDS | 3 g |

Made to 300 ml with deionized water. Stored at room temperature and diluted to appropriate volume before use.

*Transfer Buffer*

| | |
|---|---|
| Tris Base | 3.03 g |
| Glycine | 14.4 g |
| Methanol | 200 ml |

Tris and glycine were mixed in 200 ml methanol and adjusted to a volume of 1000 ml with the addition of deionized water and stored at 4°C.

*Coomassie Blue Stain*

0.1% Commassie blue R-250
40% methanol
10% acetic acid
50% deionized water

Stain for ½ hour with gentle rocking.

*Destain Solution*

40% methanol
10% acetic acid
50% deionized water
Destain with several changes for at least one hour.

*TBST-Solution*

100 mM Tris HCl
0.9% NaCl
0.1% Tween 20

Stored at room temperature.

# APPENDIX F

## RT-PCR BUFFERS

Based on the methods detailed in Current Protocols in Molecular Biology, volume 1, chapter 2.5 (Aussubel 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 Sample Loading Buffer*

20% Ficoll 400
0.1% SDS
0.25% bromphenol blue
0.25% xylene cyanol

Add 5 µl to 50 µl PCR sample reaction mix.

*10x TBE*

| | |
|---|---|
| Tris HCl | 108 g |
| Boric acid | 55 g |
| 0.5 M EDTA | 40 ml |

Bring up to 1 litre volume with deionized water.

*2% Agarose Gel*

| | |
|---|---|
| H2O | 135 ml |
| Agarose | 3 g |
| 10x TBE | 15 ml |
| Ethidium bromide | 4.5 µl |
| | 150 ml |