EXPERIMENTAL CARRAGEENAN MODEL OF ULCERATIVE COLITIS TO EXPLORE THE ROLE OF INFLAMMATION AND DIETARY LIPIDS IN THE INITIATION AND POST-INITIATION STAGES OF COLON CARCINOGENESIS

BY

NATASA SABLJIC

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree of

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Human Nutritional Sciences
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FACULTY OF GRADUATE STUDIES

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Table of contents

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List of abbreviations

AA	arachidonic acid
ACF	aberrant crypt foci
AIN-93	American Institute of Nutrition-93 diet
ANOVA	analysis of variance
AOM	azoxymethane
CAR	carrageenan
COX	cyclooxygenase
DHA	docosahexaenoic acid
DMH	1,2-dimethylhydrazine
FA	fatty acid
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneally
EPA	eicosapentaenoic acid
H&E	hematoxylin and eosin staining
HFB	high fat beef tallow
HFC	high fat corn oil
HFO	high fat olive oil
HFF	high fat flaxseed oil
LFC	low fat corn oil
NSAID	nonsteroidal anti-inflammatory drugs
PBS	phosphate buffered saline
PG	prostaglandin
PUFA	polyunsaturated fatty acid
s.c.	sub cutaneous
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel
SE	standard error of the mean
TNFα	tumor necrosis factor alpha
TX	thromboxane
UC	ulcerative colitis
UC-CRC	ulcerative colitis-associated colorectal cancer
wk	week

List of figures

List of tables

Abstract

The main objectives of a present study were: 1) To sequentially analyze the inflammatory effect of carrageenan (CAR) in rat colonic mucosae to determine the duration required to produce morphological and biochemical changes. 2) To assess the effect of the duration of CAR feeding before and concurrent with the administration of carcinogen injection on the sensitivity of colonic mucosae to azoxymethane (AOM) induced colon carcinogenesis. 3) To understand the effect of high fat diets, varying in fatty acid composition, on the ability of CAR to augment the formation of colonic preneoplastic lesions. 4) To assess whether high and low content of ω-6 polyunsaturated fatty acids (PUFAs) in a diet affects the levels of pro-inflammatory molecules in carcinogen treated animals differently in the presence of CAR.

These objectives were met by conducting three experiments. In experiment 1, female Sprague Dawley rats were fed a low fat corn oil diet (LFC) without CAR for 6 weeks or with CAR (LFC+) for 1, 2, 4, and 6 weeks. Colonic mucosa were evaluated at each time point for morphological evidence of inflammation and change in the levels of COX-2, iNOS and TNF-α. Within 2 weeks of CAR feeding, lymphocyte infiltration and increased number of lymph nodules were noted, followed by focal epithelial erosion and ulceration. Immunologically reactive iNOS and TNF-α increased, while COX-2 decreased, in the LFC+ group compared to LFC group.

In the second experiment rats were fed LFC diet for 6 weeks or LFC+ diet 2, 4, and 6 weeks before or after AOM injection. A significant increase in the number and growth of ACF occurred 6 weeks pre-initiation and 2 weeks post-initiation CAR feeding.

In the third experiment rats were fed low or high fat diets with or without CAR for 2 weeks, injected with AOM, and continued on the same diet for additional 4 weeks. Their colons were assessed for ACF. The heightened risk of colon cancer by CAR feeding was evident only in LFC+ group and assessed as the total number of ACF in HFF+ (high fat flaxseed oil) diet group, but not in the high fat diet groups containing corn oil (HFC+), olive oil (HFO+), or beef tallow (HFB+). The HFC+ diet had significantly lower number of ACF than HFC diet. The HFF diet reduced both, the number and growth of ACF.

This study conclusively demonstrated that CAR feeding exerts a cocarcinogenic effect, however, this effect was noted only in the low fat but not in the high fat diet groups. In the future long-term studies should be conducted to investigate the effect of CAR on tumor incidence and phenotype. In addition, the biochemical and molecular bases for interaction among high fat diets, inflammation and carcinogenesis should be explored.

Review of the literature

(Chapters 1, 2, and 3)

CHAPTER 1

1.0. Cancer

Cancer, "an evolutionary drama played out in the body's various parts", has been known to human societies since ancient times (Gopaul, 2004). However, since most cancers develop later in life, it does not come as a surprise that the number of people affected by the disease began noticeably to increase at the end of 19th century together with prolonged life expectancy, which was brought by medical advances. Moreover, the larger cancer incidence in the developed countries, the striking increase in the incidence among migrants from low risk to high-risk regions, and the rapid increase over time within certain countries, indicate that some aspect of lifestyle or environment play important causative roles.

Given the current trends, at least one in four men and one in five women will die from some sort of cancer (Franks and Teich, 2001). With an increase of ageing population, globalization and "westernization", cancer is expected to replace cardiovascular disease as the leading cause of mortality during the first half of 21st century (Hiatt and Rimer, 1999). Thus the importance of cancer control, in particular, its prevention, is immense.

1.1. Colon

1.1.0 Anatomy and physiology

The large intestine constitutes the terminal part of the gastrointestinal tract (GIT) and is in humans on average 1.5 metres in length. Anatomically the large intestine is divided into

three main sections: cecum including the appendix, colon, and rectum with the anal canal. Its primary function is to absorb water, electrolytes, and some vitamins from the chymes received from the small intestine and to compact, store and eliminate undigested nutrients and indigestible components. The only secretion of importance is mucus, which acts as a lubricant for transport of intestinal contents (Gartner and Hiatt, 1997; Sizer and Whitney, 1997).

The colon, composed of the ascending, transverse, descending and sigmoid part, accounts for almost the entire length of the large bowel. The ascending colon begins where the small bowel attaches to cecum (ileocecal valve) and extends upward on the right side of the abdomen progressing into the transverse colon, which goes across the body to the left side. The descending colon attaches onto the transverse colon and continuous downward on the left side. The last part or the distal colon is called sigmoid because of its S-shape. The sigmoid colon joins the rectum, which, in turn, joins the anus (Figure 1.1) (Gartner and Hiatt, 1997).

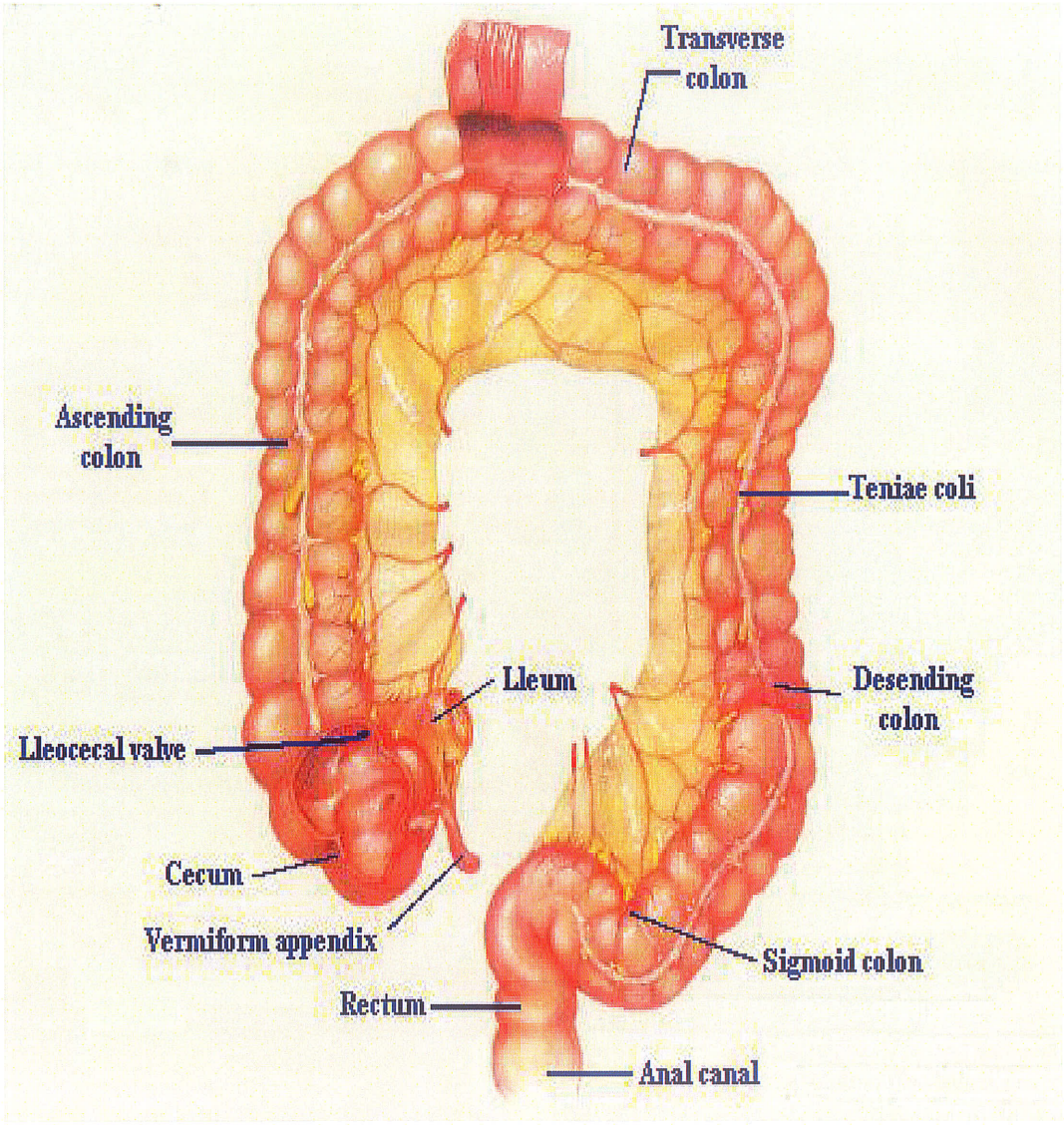
1.1.1. Histology

Histologically colonic wall is made up of four layers of tissue: the mucosa, submucosa, muscularis externa and serosa. Colon cancer generally starts in the mucosa, the innermost layer, and can grow through some or all of the other layers.

1.1.1.0. Mucosa

The mucosal lining consists of the columnar epithelium shaped into straight crypts. These finger-like shaped invaginations of mucosal epithelium provide protected

Figure 1.1. Anatomy of the large intestine.
(http://8adigest.tripod.com/digestivesystem/id3.html)


Transverse colon
Ascending colon
Teniae coli
Lleum
Desending colon
Lleocecal valve
Cecum
Vermiform appendix
Sigmoid colon
Rectum
Anal canal

pockets for special cellular functions (Gartner and Hiatt, 1997). Deeper into colonic wall crypts are supported by the lamina propria (connective tissue rich in vascular and lymphatic vessels, and some plasma cells, lymphocytes, eosinophils, macrophages, mast cells, smooth muscle cells and fibroblasts), which is in turn surrounded by the muscularis mucosae (the layer composed of an inner circular and an outer longitudinal sheet of smooth muscle) (Cotran et al., 1999; Gartner and Hiatt, 1997) (Figure 1.2A).

1.1.1.1. Crypts

Colonic crypts, sometimes called "intestinal glands" or crypts of Lieberkühn, are predominantly composed of three types of epithelial cells: absorptive, goblet and enteroendocrine (Gartner and Hiatt, 1997) (Figure 1.2B). It is believed that common, undifferentiated, stem cells located at the base of the crypts regenerate epithelium, giving rise to new cells from all three lineages (Kirkland, 1988). The process of regeneration starts at the lower two-third of the crypt with proliferation of stem cells. Newly formed cells then undergo the process of differentiation while moving upward toward the luminal surface. Once they reach the top of the crypt, the cells are mature and ready to replace the absorptive or secretory cells that underwent apoptosis and had been sloughed off and shad into the colonic lumen (Gartner and Hiatt, 1997).

The entire surface of the colonic epithelium is replaced every 3 to 8 days (Cotran et al., 1999). This rapid regeneration provides remarkable capacity for repair but also makes the colonic epithelium particularly vulnerable to all agents that interfere with cell replication.

Figure 1.2A. Histology of human colonic mucosa. A longitudinal section H&E stained. Magnification x65. (http://www.hm.harvard.edu)

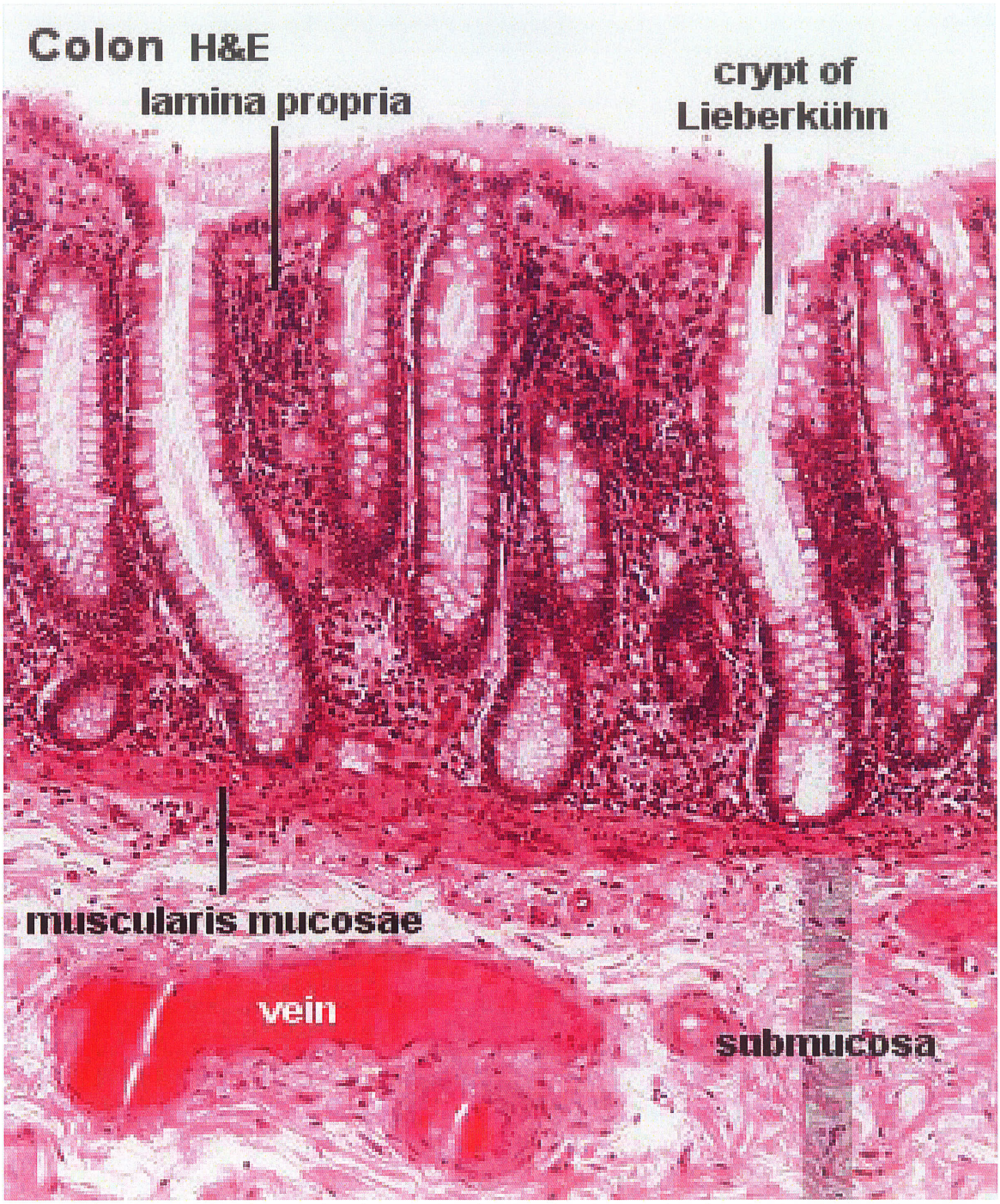
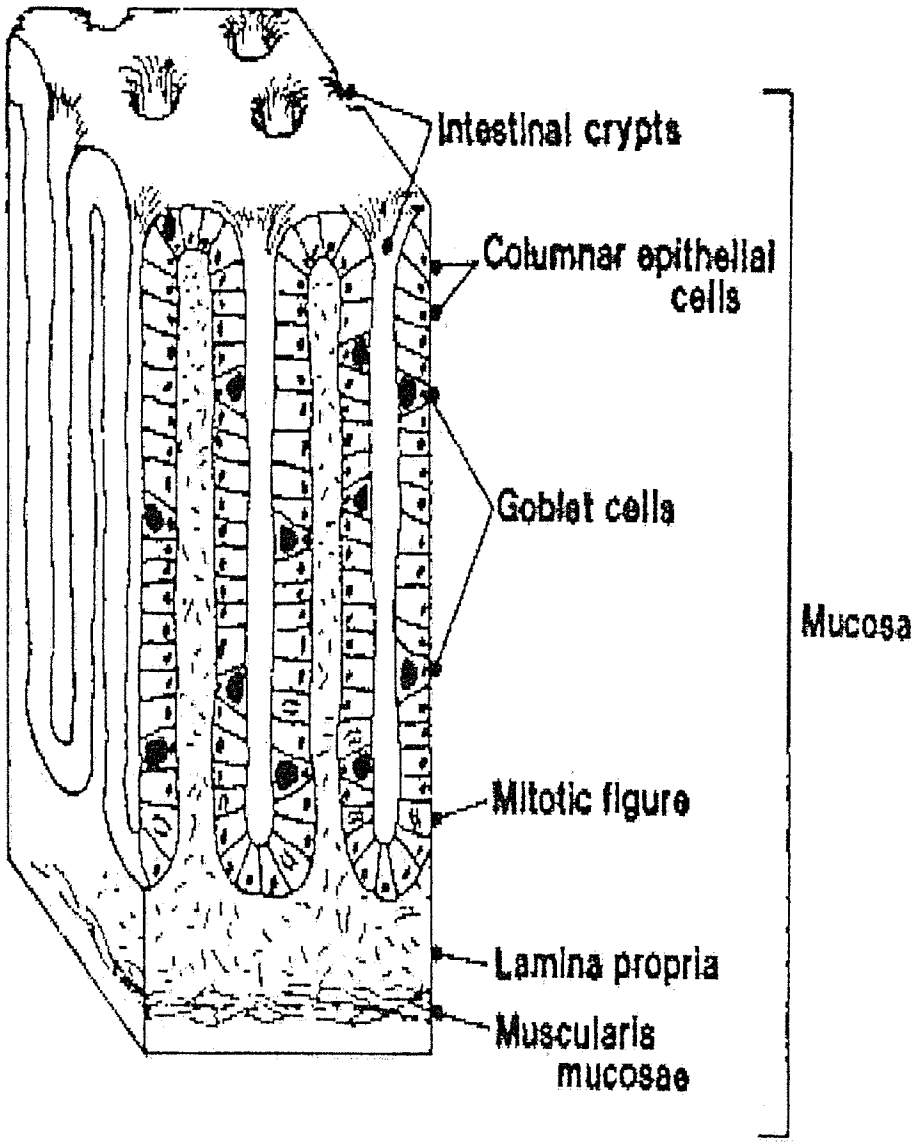

Colon H&E
lamina propria
crypt of
Lieberkühn
muscularis mucosae
vein
submucosa

Figure 1.2B. Structure of colonic mucosal crypts. Schematic representation

(http://www.uwgi.org/gut/colon.htm#C)


Intestinal crypts
Columnar epithelial cells
Goblet cells
Mucosa
Mitotic figure
Lamina propria
Muscularis mucosae

1.1.1.2. Lymphoid tissue

Lymphoid tissue occurs in mucosa all along the GI tract, where it is sometimes referred to as GALT (gut-associated lymphoid tissue). GALT is considered the largest subdivision of MALT (mucosa-associated lymphoid tissue). The role of lymphoid tissue is to react against foreign proteins (antigens) either by direct attack (cellular immunity) or by production of antibodies (humoral immunity).

Lymphocytes are either scattered in the epithelium of colonic mucosa (at a ratio1: 6 with epithelial cells) or form solitary lymphoid nodules in the lamina propria (Gartner and Hiatt 1997; Johnson, 1987). The lymphoid nodules are composed of B lymphocytes surrounded by a looser region of T lymphocytes and numerous antigen-presenting cells. At the center of each lymph nodule is a "germinal center" where the lymphocytes proliferate. The connective tissue around a lymph nodule is usually heavily infiltrated with lymphocytes migrating to and from the germinal center (Gartner and Hiatt, 1997).

The most characteristic feature of gut-associated lymphoid tissue is the presence of clusters of lymph nodules. These longitudinal aggregations of 30 to50 nodules, which bulge upward toward the lumen, are known as lymphoid follicles or Payer's patches (Targan and Shanahan, 1994). It is believed that antigen entry to the gut is via follicle-associated epithelium known as M cells. These specialized cells initiate the mucosal immune reaction by capturing antigens from the lumen and presenting them to lymphocytes of Payer's patches (Gartner and Hiatt, 1997).

1.2 Colon Cancer

Even though the epidemiology of colon and rectal cancers is somewhat different and ideally these diseases should be considered separately (Franks and Teich, 2001), it is important to note that since there is a close anatomical, physiological and histological connection between colon and rectum, they are very often referred and investigated together as colorectal cancer. Again, based on similarity and convenience, the present study includes both, colon and rectum, in its investigation.

1.2.0. Epidemiology and risk factors

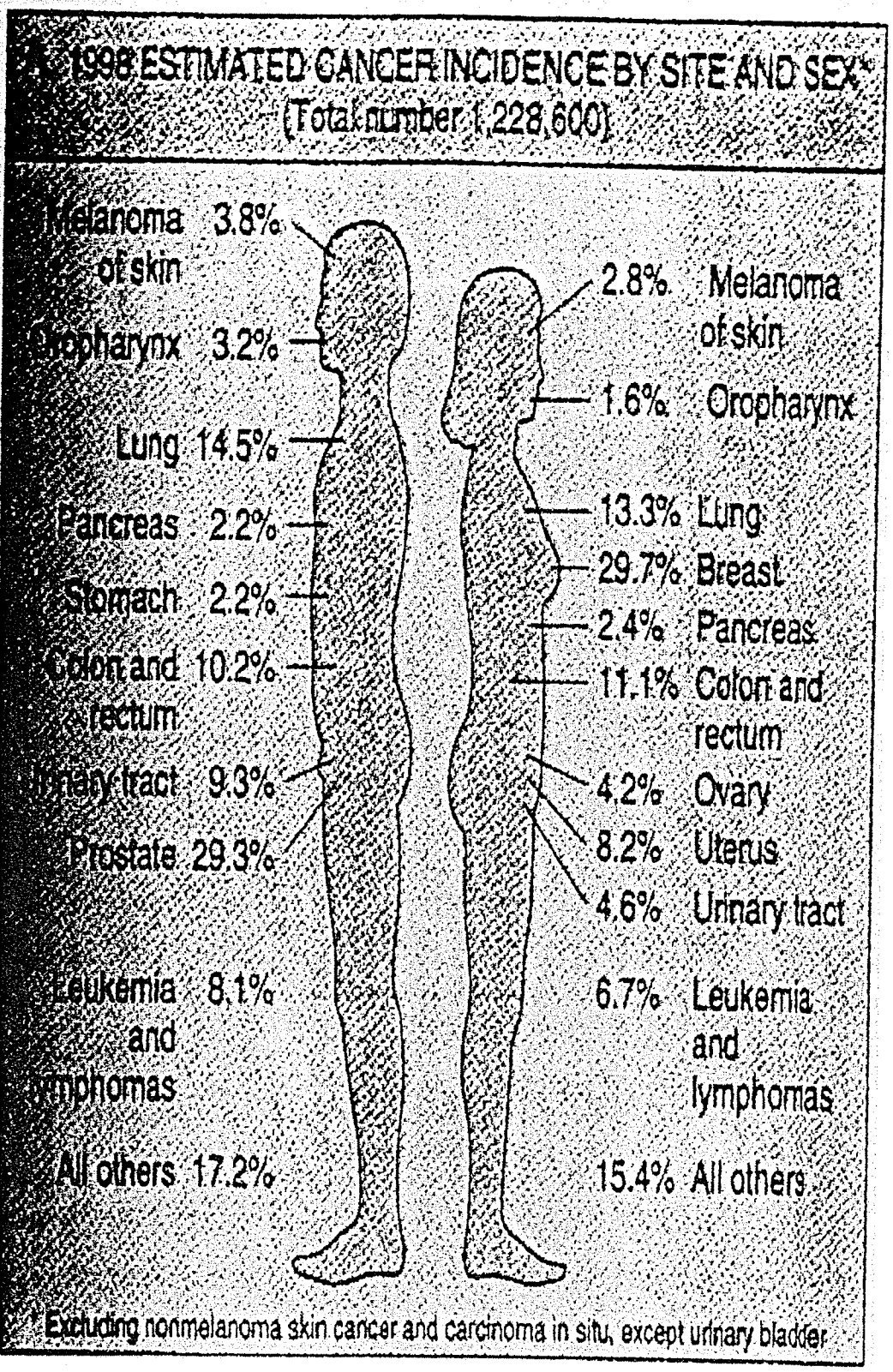
Colon cancer is the third most frequently diagnosed cancer and the second leading cause of cancer mortality among man and women in the developed world (Figure 1.3) (Franks and Teich, 2001; Smalley and DuBois, 1997). Although, it can occur at younger ages, even, in rare cases, in the teens, this disease predominantly affects people over the age of fifty.

Even though overall incidence of colon cancer shows a regular increase of 3-7% annually (Roynette et al., 2004), it started to fall at younger ages in the late 1980s, and it has decreased since by more than 1% annually in the younger age group (Franks and Teich, 2001; Harvard Health Online, 2000). One possible explanation for this trend might lay in dietary and/or lifestyle changes initiated by educational campaigns to reduce the risk of colorectal cancer, cardiovascular and other metabolic diseases, which would be expected to affect young people first.

The exact causes of colorectal cancer are unknown, but both, inherited (genetic)

Figure 1.3. Cancer incidence by site and sex. (Cotran et al., 1999).

It is estimated that there were 1,228,600 new cases of cancer in 1998 in the US. Colon cancer was the third most frequently cancer with an incidence rate of 10.2% in man subsequent to prostate and lung cancers and an incidence rate of 11.1% in women subsequent to breast and lung cancers.


1998 ESTIMATED CANCER INCIDENCE BY SITE AND SEX*
(Total number 1,228,600)
Melanoma of skin 3.8%
Oropharynx 3.2%
Lung 14.5%
Pancreas 2.2%
Stomach 2.2%
Colon and rectum 10.2%
Urinary tract 9.3%
Prostate 29.3%
Leukemia and lymphomas 8.1%
All others 17.2%
2.8% Melanoma of skin
1.6% Oropharynx
13.3% Lung
29.7% Breast
2.4% Pancreas
11.1% Colon and rectum
4.2% Ovary
8.2% Uterus
4.6% Urinary tract
6.7% Leukemia and lymphomas
15.4% All others
* Excluding nonmelanoma skin cancer and carcinoma in situ, except urinary bladder

and environmental factors have been identified as risk factors. A small proportion of colon cancer is due to inheritance of mutations in certain genes. The most common hereditary colon cancer syndromes are familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC). It is estimated that these dominantly inherited conditions might account for up to 5-10% of all large bowel malignancies (Ponz de Leon and Roncucci, 2000). It has also been shown that the immediate family members of patients with sporadic colon cancer, not caused by inheritance of a mutated gene but by mutations arising in somatic cells, are at increased risk (two to threefold) of developing the disease (Hardy et al., 2000). Thus, why some families and races are more prone to development of colon cancer cannot be always explained by an obvious genetic predisposition, but rather seems to involve on the combination of various genetic and environmental factors.

Differences in dietary practices and life-styles in different geographic regions have been associated with an altered risk for developing colorectal cancer (Franks and Teich, 2001). Environmental factors, such as sedentary lifestyle, obesity, inflammatory bowel disease (IBD), and dietary habits including increased intake of red meat, saturated fat, and alcoholic beverages, as well as decreased intake of fibre and protective micronutrients, are considered to heighten risk for colon cancer (Ponz de Leon and Roncucci, 2000; Willett, 2000). Accepting the concept that colon cancer is a preventable disease research efforts are directed toward identity of cancer preventive agents and strategies, so along that line the role of dietary fat and IBD as the risk factors involved in the development of colon cancer will be further discussed later.

1.2.1. Carcinogenesis

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. Adenocarcinoma, an epithelial neoplasm, is the most frequent malignity seen in the colons of humans as well as in laboratory animals treated with colon specific carcinogens (Pretlow and Pretlow, 1997). It is generally believed that the transformation of the normal colonic epithelial cells into cancer cells is a multistage process that starts with specific genetic alterations (mutations) induced by the carcinogen. During this first stage, referred to as an initiation stage, irreversible changes in DNA take place causing decreased responsiveness of initiated cells to the inter/intra cellular signals known to maintain normal cellular growth and functions (Roynette et al., 2004; Curtis, 1991).

The next stage is a promotion stage with continuous clonal selection and propagation of initiated cells (Farber, 1995). After a series of proliferation all cells inside one crypt might get altered forming a histological structure known as aberrant crypt focus (ACF) (Bird et al., 1989). It is believed that additional genetic events then lead to a development of a small intra-epithelial benign structure known as adenoma (polyp or flat/non-polypoid), which is characterized by the presence of low or high-grade dysplasia (Guindi and Riddell, 2001).

Highly dysplastic cells further change into an early neoplastic clone of cells, which in turn, during a progression stage, develops into a fully malignant phenotype via both genetic and epigenetic mechanisms turning adenoma into a cancer (Curtis, 1991) (Figure 1.4).

This process of malignant transformation, known as colon carcinogenesis,

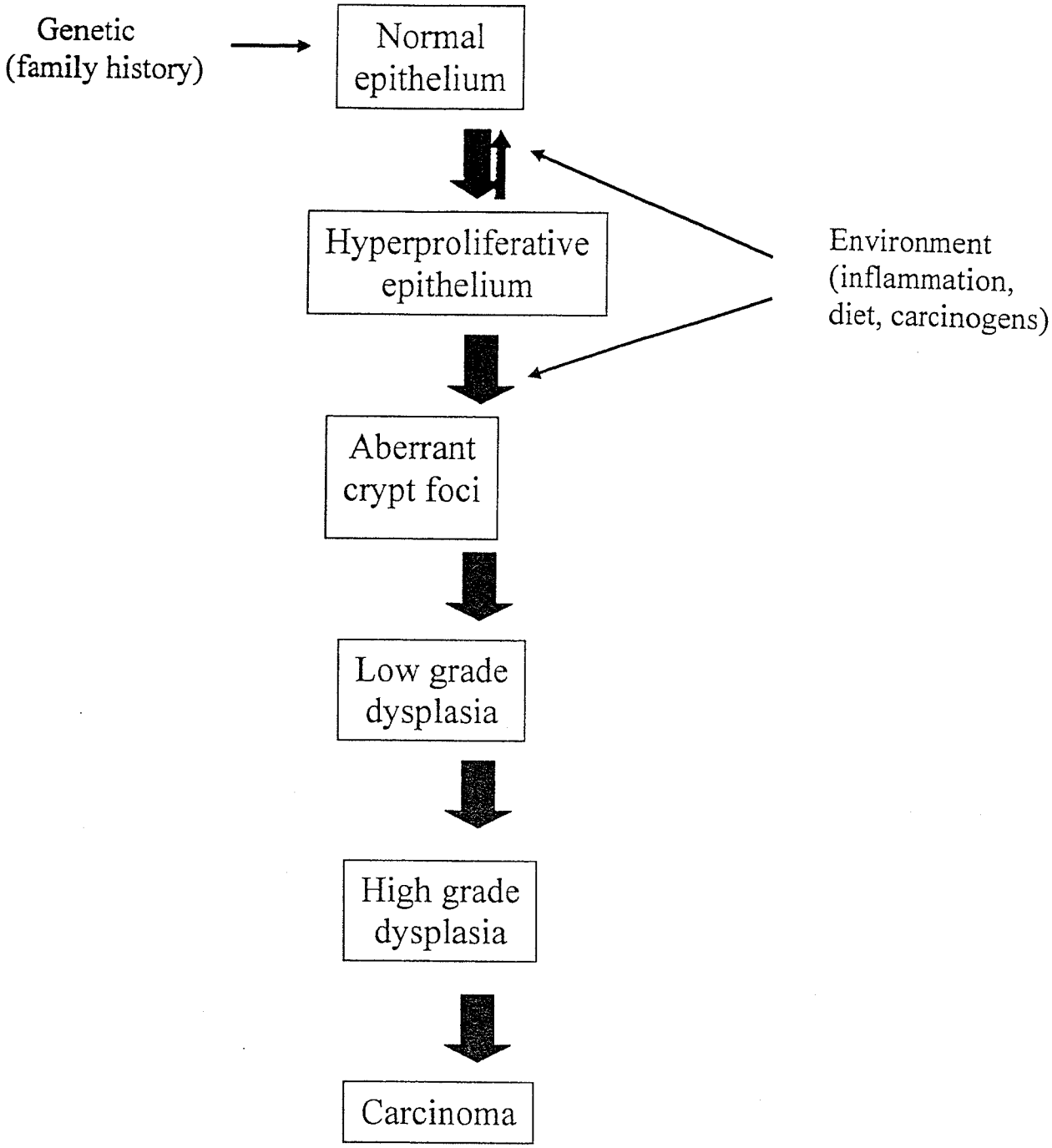
develops slowly over a period of many years (5 to 40) and involves multiple genetic alterations (as many as 20 genes may be involved), formation of oncogenes and inactivation of tumor suppressor genes (Curtis, 1991; Roynette et al., 2004). Although normal colonic epithelium is polyclonal in nature, it has been proposed that during carcinogenesis each subsequent mutational event provides a growth advantage for a single cell over the other tumor cells suggesting monoclonal nature of colonic neoplasia (Chang, 1984).

Once fully formed, cancer cells can invade nearby tissues and spread (metastasize) through the bloodstream and lymphatic system to other parts of the body. Therefore, the sooner colorectal cancer is detected and treatment begins, the lower is the risk of its spread and the chances for survival are imporoved.

1.2.2. Aberrant crypt foci (ACF)

Despite all medical and pharmacological advances early diagnosis and intervention is still the key to curative treatment and to prevention of cancer-related deaths. Unfortunately, it is estimated that of all colon cancer cases less than 50% are detected in their early, most curable stages (Harvard Health Online, 2000). Hence, there is an enormous importance to identify and investigate early lesions and conditions under which they undergo malignant transformation. A thorough understanding of early lesions and related markers would help to develop and implement strategies that would prevent colon cancer evolution. Through sequential morphological analysis of carcinogen-treated rodents Lipkin and Deschner (1976), Richards (1997), and Chang (1984) identified crypts with distinctly altered proliferative patterns and proposed that such crypts represent one

Figure 1. 4. Key events in a multi-step process of colon carcinogenesis. (Modified from Hardy et al., 2000.)


Genetic
(family history)
Normal epithelium
Hyperproliferative epithelium
Environment
(inflammation,
diet, carcinogens)
Aberrant crypt foci
Low grade dysplasia
High grade dysplasia
Carcinoma

of the early precursor lesions from which colon cancer evolves. In 1987, Bird identified lesions in whole-mount colons of carcinogen treated rodents by developing a simple, quick method using light-microscopic examination and termed them aberrant crypt foci (ACF). Pretlow at al. (1991) and Roncucci et al. (1991) using Bird's method described ACF in macroscopically normal colonic human mucosa. They found that the frequency of ACF was higher in patients with colon cancer than in those without cancer or predisposing conditions for colon cancer, and was highest in patients with familial polyposis coli. Moreover, the frequency of ACF in a patient with Chron's disease was similar to those in patients with colon cancer (Pretlow et al., 1991).

The above-mentioned findings led to the unanimous conclusion that the presence of ACF in colonic mucosa increases the risk of colon cancer development and initiated the hypothesis that these lesions may represent the earliest histologically identifiable preneoplastic lesions. Today it is believed that ACF are precancerous lesions and that a large percentage (if not all) of colorectal cancers develop from ACF (Pretlow and Pretlow, 1997).

1.2.2.0. Putative precancerous lesions

Numerous studies have supported the hypothesis that ACF are precancerous lesions. Such evidences include: 1) ACF are induced specifically by colon carcinogens (never detected in control animals) in a species, strain, and dose specific manner (McLellan and Bird, 1988; Tudek et al., 1989); 2) ACF are detected in the colons of humans with a high risk for developing colon cancer (Pretlow at al. 1991; Roncucci et al., 1991); 3) Phenotypic and genotypic characteristics of ACF fall right in the middle

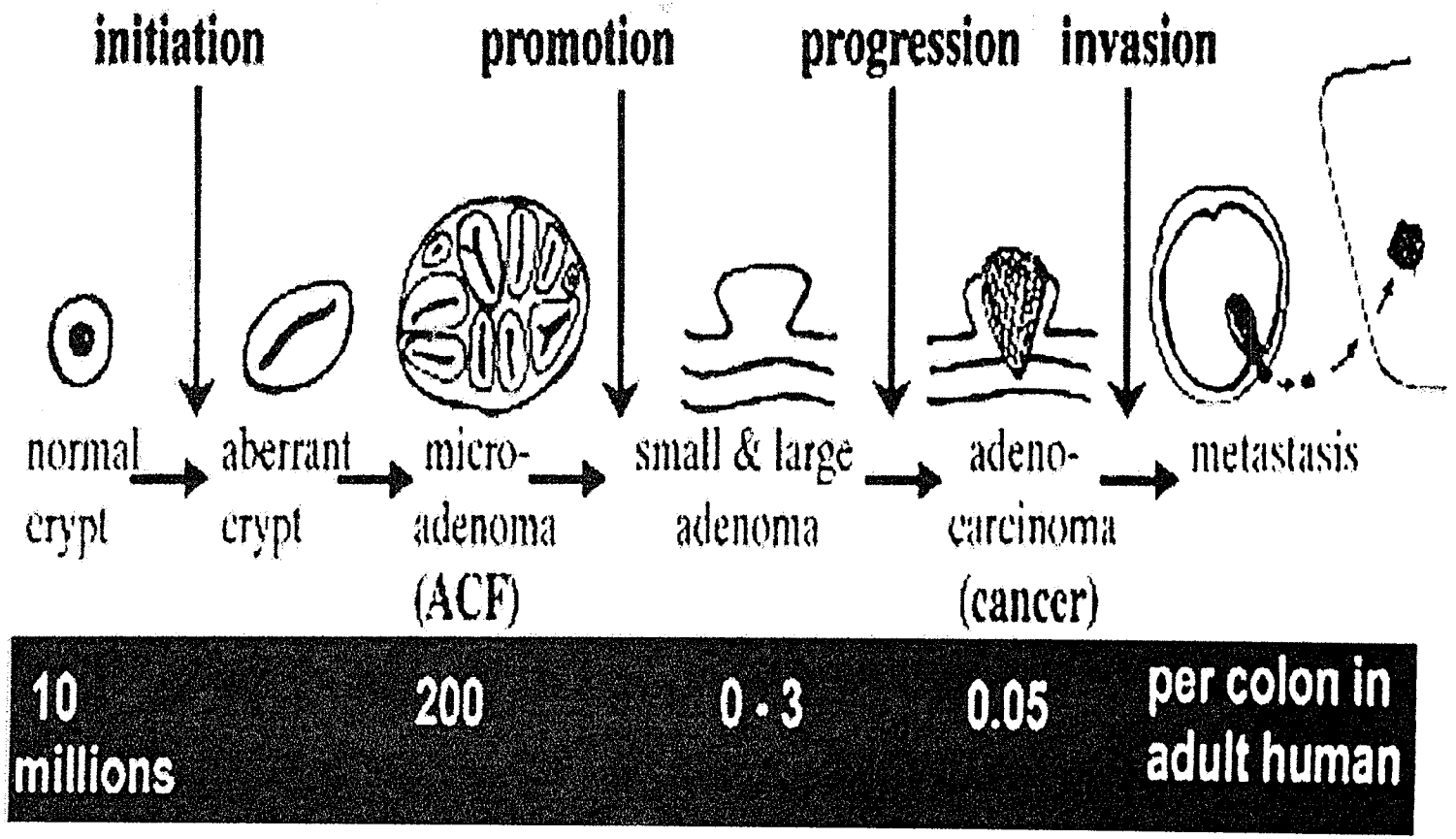
between normal and malignant tissue (Franks and Teich, 2001); 4) Dysplastic morphology, proliferative features, changes in enzymatic activity, genetic alterations in expression of cell-cycle-controlling genes, K-*ras* and somatic APC mutations, that are observed in colon cancers have been detected in some ACF (Jen et al., 1994; Smith et al., 1994; Pretlow and Pretlow, 1997), 5) The appearance of ACF can be mediated with known tumor promoters or inhibitors (Pereira and Khoury, 1991; Mclellan and Bird, 1991; Shivapurkar et al., 1992); and 6) The ACF system has generally been found to predict tumor outcome in a consistent manner (Bird, 1998) (Figure 1.5).

1.2.2.1. Number and growth features of ACF

Within 2 weeks after carcinogen treatment the first aberrant crypts foci can be observed in the colonic mucosa of rat. A focus of aberrant crypts can consist of one to several crypts and at early time points (2-4 weeks) most of the foci are composed of 1 to 3 crypts (McLellan et al., 1991; Pretlow and Pretlow, 1997). As the time after treatment with carcinogen becomes longer the number of ACF per colon increases and many of the foci expand clonally increasing in their focal size and crypt multiplicity (number of crypts per focus) (Bird et al., 1989; Bird, 1997). Moreover, as they develop, ACF start to exhibit varying grades of proliferative atypia and dysplasia (Bird, 1997).

It is interesting to note that in the same colon, ACF consisting of varying numbers of crypts and different degrees of dysplasia exist simultaneously, indicating that not all ACF develop at the same rate or perhaps even have the same potential to progress to adenocarcinomas (Bird et al., 1989; Pretlow and Pretlow, 1997). McLellan et al. (1991), investigated ACF in colons of rodents from 2 to 57 weeks after carcinogen treatment, i.e.

Figure 1.5. Aberrant crypt foci (ACF) – putative preneoplastic lesions. Diagram depicts hypothesized progression of aberrant crypt foci (ACF) into cancer trough the process of multi-step carcinogenesis. (http://corpet.free.fr/acf.html)


initiation
promotion
progression
invasion
normal crypt
aberrant crypt
micro-adenoma (ACF)
small & large adenoma
adeno-carcinoma (cancer)
metastasis
10 millions
200
0 - 3
0.05
per colon in adult human

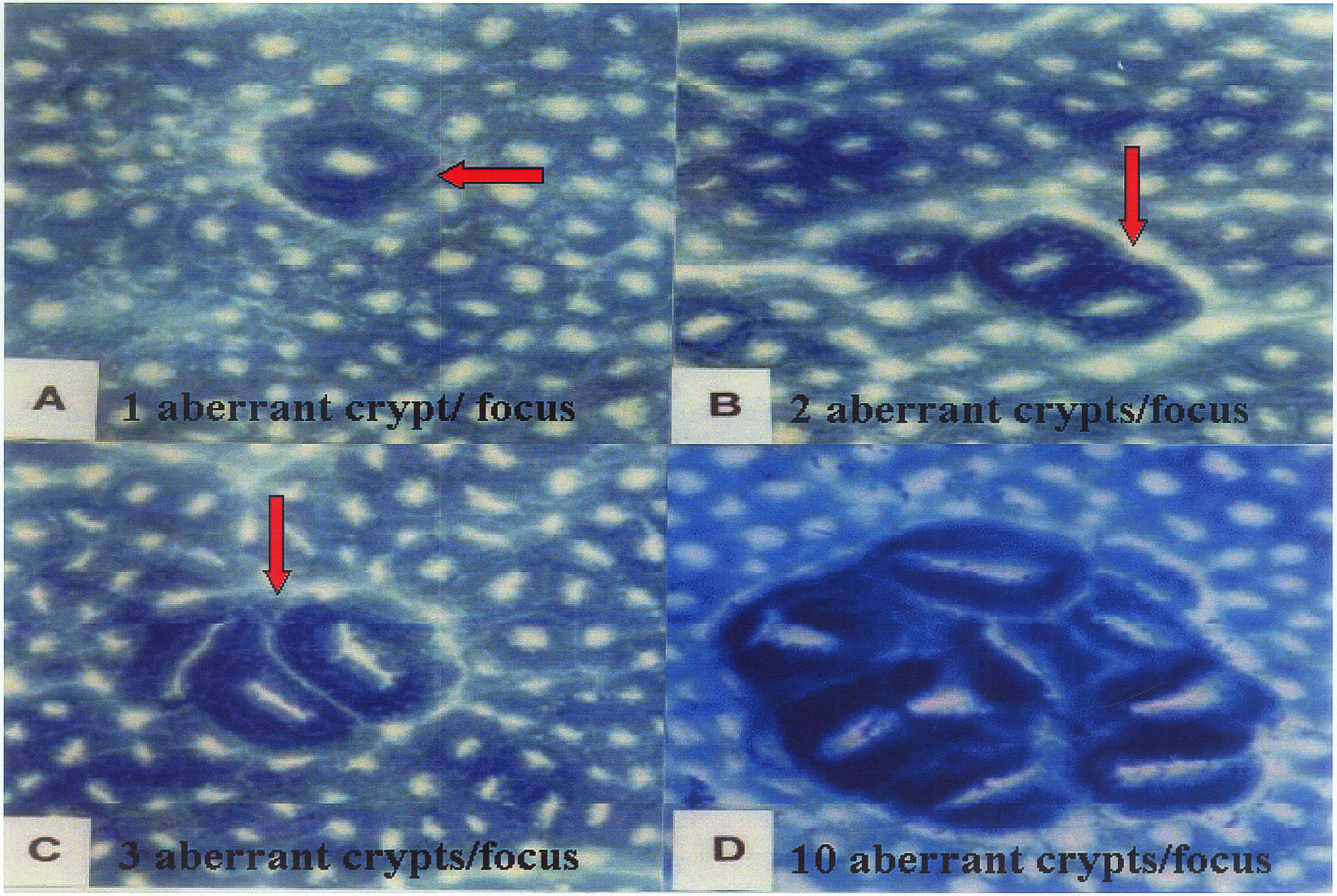
until adenocarcinomas fully developed. They observed that the number and multiplicity of ACF was changing (either increasing or decreasing) indicating that ACF are in a dynamic state and may modify (progress or regress). It was proposed that those gradually progressing lesions eventually attain complete growth autonomy as illustrated previously in the Figure 1.5. (Shirtliff and Bird, 1996).

1.2.2.2. Histology of ACF

In general, aberrant crypts contain more epithelial cells and less goblet cells than normal crypts. Depending on the degree of their alteration epithelial cells in aberrant crypts exhibit a high nuclear to cytoplasmic ratio, prominent nucleoli, cytoplasmic basophilia, loss of cell polarity and increased proliferative activity in the upper part of the crypt (Bird et al., 1989). During histologycal examination of the colonic mucosal surface of longitudinally opened, flat fixed, methylene blue-stained, unsectioned colons, crypts built of atypical cells appear as distinct focal lesions much larger than the surrounding normal crypts, with luminal openings that are elliptical rather than round, a thicker epithelial lining and a greater pericryptal area which separates them from adjacent normal crypts (McLellan et al., 1991; Bird et al., 1989) (Figure 1.6).

The parameters generally used to assess the number and growth features of ACF include: 1) the number of ACF (the mean total number of ACF/colon in a group), 2) crypt multiplicity (the mean number of aberrant crypts/focus/colon of all ACF found in a group), 3) the distribution of foci (the mean number of ACF/each segment along the length of the colon in a group), and 4) the size of the focus (area occupied by each ACF) (Bird, 1998). The two parameters found to be most reliable in the predicition of

Figure 1.6. Topographic views of ACF with different crypt multiplicity (number of crypts in each focus) in whole-mount methylene blue stained colon of a carcinogen treated rat. Figure 1.6. A) depicts a single crypt focus surrounded by normal crypts. Figures 1.6. B, C, and D) depict aberrant crypts foci with different crypts multiplicity - two, three and ten crypts per focus respectively. Note the increased size, elliptical lumen, thicker epithelial lining and greater pericryptal area of aberrant crypts. Magnification x400.


A
1 aberrant crypt/ focus
B
2 aberrant crypts/focus
C
3 aberrant crypts/focus
D
10 aberrant crypts/focus

tumorigenicity are the number of ACF and crypt multiplicity (Shirtliff and Bird, 1996).

1.2.2.3. Value of ACF as a biomarker of colon carcinogenesis

Technically, it is difficult to study ACF in human colons; however, in animal-based studies the ACF system have proved superior as it is the only biomarker that provides a quantitative assessment at precancerous stages (Bird and Good, 2000). Research done on carcinogen-treated rodents has shown that sequential analysis of ACF with respect to their number, size, morphological atypia, proliferative pattern and histochemical properties may be used as endpoint markers in investigations of early cellular and molecular changes in colorectal carcinogenesis. The ACF system has been extensively utilized in the identification of tumor promoters and inhibitors in the environment as well as in understanding the mechanisms involved in the stepwise progression of the cancer development (Bird and Good, 2000).

In the present research, evaluation of ACF will be used to further our understanding of the carcinogenic process as it occurs under inflammatory conditions in the rodent colon, and will provide a basis for investigating the role of dietary lipids in the aetiology of colitis-associated colon cancer.

1.2.3. Azoxymethane (AOM)

A large number of agents, including radiation, chemical carcinogens and oncogenic microbes, may cause genetic damage and induce malignant transformation of cells (Franks and Teich, 2001). Chemical carcinogens, either natural or synthetic, are most frequently used to induce colon cancer in animal experiments. These compounds

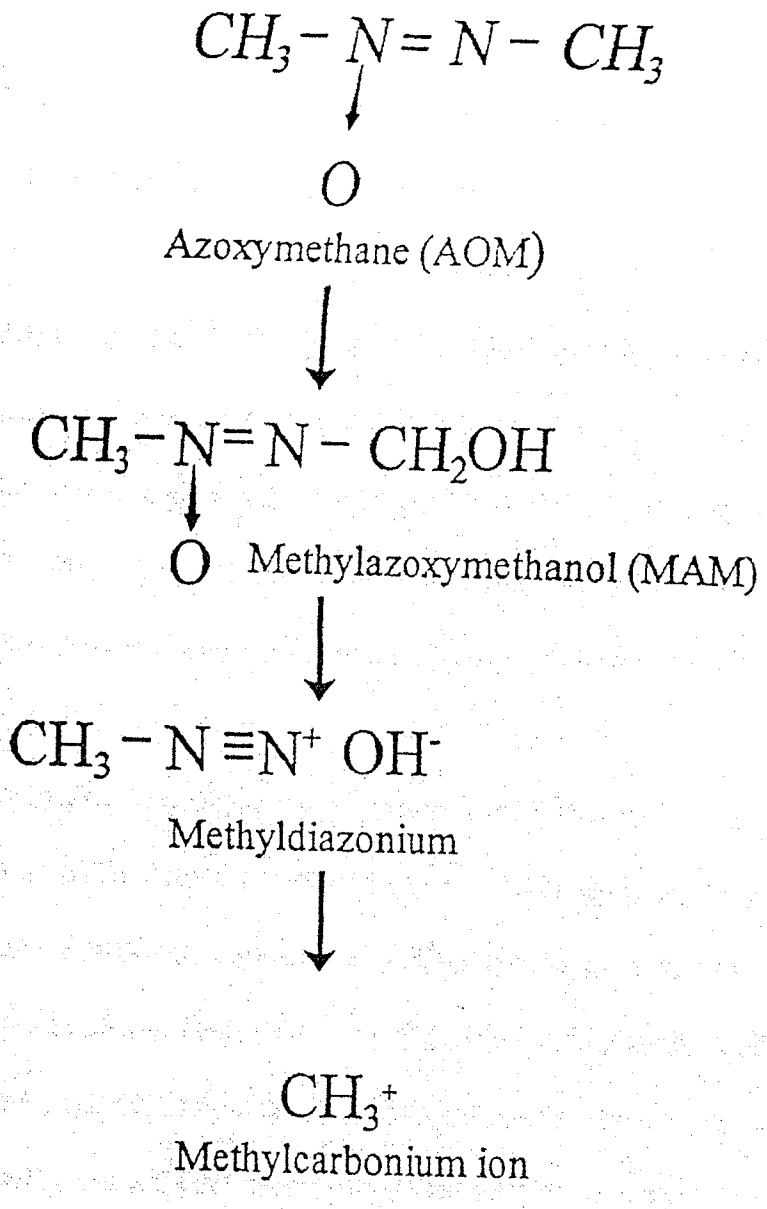
depending on their structure can act directly, or require metabolic conversion *in vivo* (procarcinogens) in order to induce neoplastic transformation (Cotran et al., 1999). Procarcinogens, 1,2-dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM), are the most popular among researchers working with rodent models (Bird, 1998). DMH and AOM are hydrazine compounds which are metabolized *in vivo* first to methylazoxymethanol (MAM) and eventually to one of the final products, a potent alkylating agent methyldiazonium, capable of methylating DNA, RNA, or proteins of colonic epithelial cells (Greene et al., 1987) (Figure 1-7). Laqueur and Spatz first identified MAM as a specific colonic carcinogen in 1963, after feeding rats with its natural source, cycad flower.

In contrast to the preparation of DMH which requires a great deal of care, AOM is commercially obtained in a liquid form and easily diluted to the appropriate concentration in sterile saline. AOM is usually administrated to rats at a dose level of 10-20 mg/kg body weight either intraperitoneally (i.p.) or subcutaneously (s.c.) (Bird, 1998). The s.c. route of administration appears to yield fewer tumors in the small intestine than i.p. route (Bird, 1998); hence, in our laboratory subcutaneous administration of AOM is a preferred way to induce tumors.

1.2.4. Animal models

Rodent models (rats and mice) are commonly used to study the toxicity, carcinogenicity, or cancer modulating ability of a compound (Bird, 1997). AOM-induced precancerous lesions and tumors in rodents share many histopathologic characteristics

Figure 1.7. Metabolic activation of the colon procarcinogen azoxymethane (AOM). (Adapted from Greene et al., 1987.) One hour after injection AOM reaches the liver where it is metabolized to methylazoxymethanol (MAM) by N-hydroxylation. MAM is then metabolized in the liver and/or extra hepatic tissues (colon) into methyldiazonium which is a reactive alkylating agent capable of methylating DNA.


$CH_3 - N = N - CH_3$
O
Azoxymethane (AOM)
$CH_3 - N = N - CH_2OH$
O
Methylazoxymethanol (MAM)
$CH_3 - N \equiv N^+ OH^-$
Methyldiazonium
CH_3^+
Methylcarbonium ion

with those observed in humans (Franks and Teich, 2001). Precancerous stages are well defined, aberrant crypts precedes development of adenomas, which increase in number with both, carcinogen dosage and time (Bird, 1998). At the molecular level there are similarities and differences between human and AOM-induced tumors. Unlike sporadic human tumors, AOM- induced tumors are seldom mutated (15%) at the adenomatous polyposis coli (APC) gene and never mutated at the p53 gene. However, like human tumors, AOM- induced tumors are often mutated on *K-ras* and beta-catenin genes and show microsatellite instability (Bird et al., 1985).

Rodents treated with AOM appear to be especially suitable to study the factors that influence, promote, or inhibit development of colon cancer from its earliest stages.

1.2.4.0. Mice versus rats

Not all species and strains of rodents are equally receptive to AOM, and while mice require multiple injections of carcinogen in order to develop colonic tumors, rats appear to be more susceptible requiring only one. Moreover, assessing two strains of rats, probably most commonly utilized for experimental purposes, Sprague-Dawley are more susceptible to AOM than the F344 strain (Nauss et al., 1987).

Studies conducted on rats concluded that the number and growth features of ACF can be determined within 4-8 weeks after AOM administration, whereas for an assessment of fully developed tumors a period of 24-28 weeks is needed (Greene et al., 1987; Bird, 1997). Five to ten rats per group were found to be sufficient for investigating early lesions of colon cancer development (Bird, 1997).

CHAPTER 2

2.0. Ulcerative colitis (UC)

2.0.0. Inflammatory bowel disease (IBD)

Ulcerative colitis (UC) and Chron's disease (CD) are two related chronic inflammatory diseases of the gastrointestinal tract denoted together as inflammatory bowel disease (IBD). Crohn's disease, named by an American medical doctor who was among the first to describe the disease, can affect any region of the gastrointestinal tract from the mouth to the anus and is characterized with discontinuous inflammation meaning that inflamed segments of the tissue are separated by healthy regions (Cotran et al., 1999). Inflammatory process in CD is most often transmular affecting all layers of the intestinal wall from the lamina propria to the serosa (De Dombal et al., 1993). In contrast, ulcerative colitis is characterized by continuous inflammation limited mostly to the mucosa of any part or the entire colon. In the rare cases, even though essentially UC involves only the large intestine, in UC patients with total colonic involvement (pancolitis), the lower part of the ileum may be mildly inflamed (Ghosh et al., 2000; Langholz et al., 1994) (Figure 2.1.).

Probably the most feared complication in patients with long-standing IBD is development of gastrointestinal cancer. In the literature, the higher risk of colorectal cancer development is reported among patients with UC as compared to those with CD (Pohl et al., 2000; Tsianos, 2000; Greenstein et al., 1980).

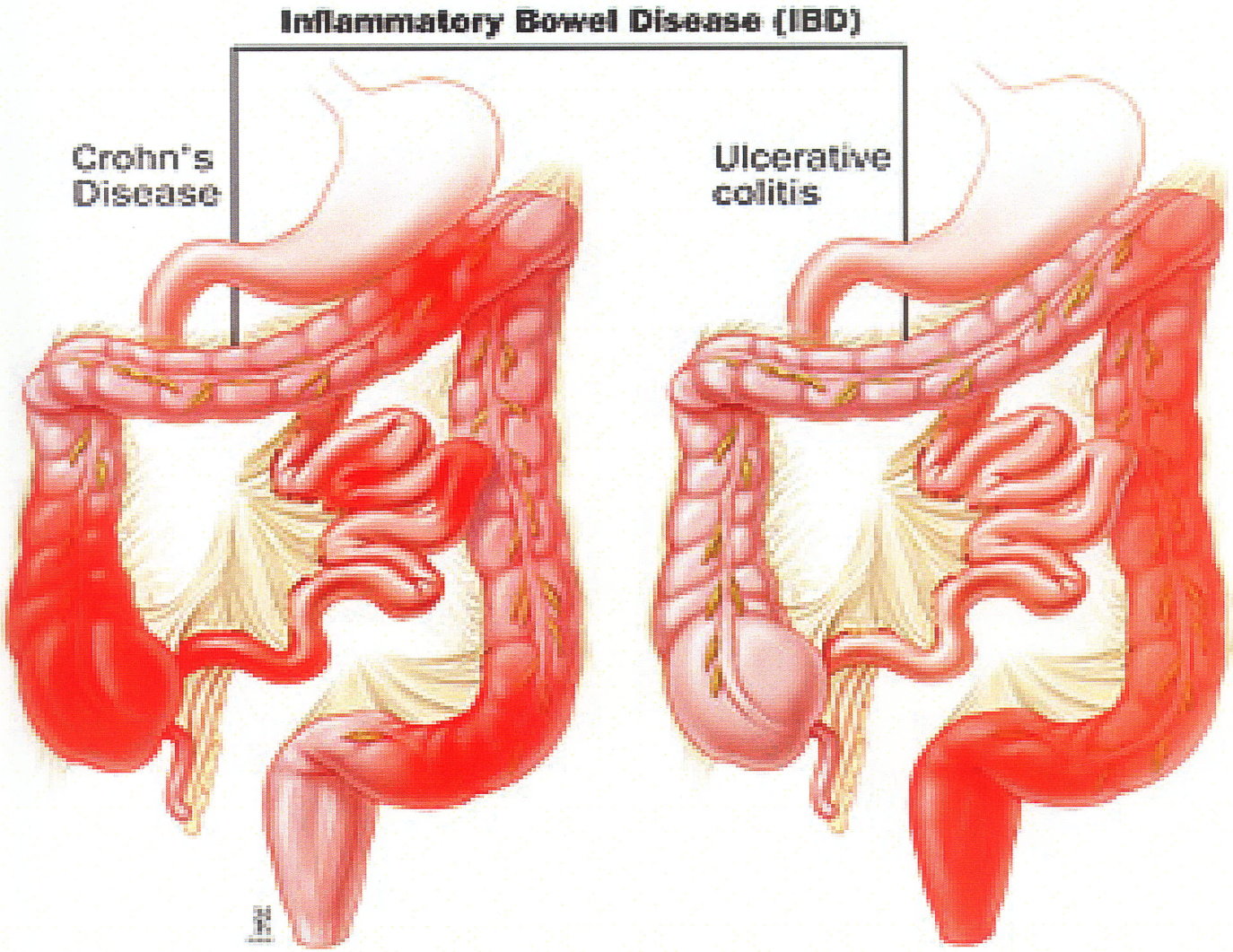
2.0.1. Epidemiology and etiology

Ulcerative colitis may be present at any age but most commonly begins in early adult life, ages 15 to 30 (Ghosh et al., 2000; Andreoli, 1997). Even though some authors have reported a slightly higher tendency of UC occurrence among women (Cotran et al., 1999; Feldman et. al., 1999; Torres et al., 2003), most of the studies have failed to find sex differences.

There is significant geographic variation in the reported incidence of ulcerative colitis. Depending on the region studied, the annual incidence of UC ranges from 0.5 to 24.5 new cases per 100.000 people (Lakatos et al., 2004; Ghosh et al., 2000; Loftus et al., 2000; Niv et al., 2000; Rubin et al, 2000; Bernstein et al., 1999; Maté-Jimenez et al., 1994; Targan and Shanahan, 1994). For yet unexplained reasons, ulcerative colitis is more common in people who live in northern climates, in developed countries, and seems to be profoundly augmented by the urban lifestyle. In Manitoba, the overall annual incidence rate reported for year 2000 was 15/100,000 and the prevalence rate was 234/100,000, which are among the highest reported in the world (Bernstein, 2004).

Several causes: genetic, infectious, environmental, immunological and psychological, have been proposed in etiology of ulcerative colitis but none of them has been proved (Schmehl et al., 2000; Cotran et al., 1999; Andreoli, 1997; Macdermott and Stenson, 1988). Recent reports showed a continuous increase in incidence rate of UC, especially in previous low incidence regions, supporting a probable causative role of environmental factors such as diet or lifestyle (Lakatos et al., 2004; Al-Shamali et al., 2003; Sood et al., 2003). The current thinking is that UC development may be influenced by the combination of genetic predisposition and environmental triggers that provoke an alteration in the way the body's immune system works.

Figure 2.1. Illustration of predominant intestinal locations of Chron's disease and ulcerative colitis. The regions affected by inflammation are portrayed with a darker shade. (www.hopkins-gi.org)


Inflammatory Bowel Disease (IBD)
Crohn's Disease
Ulcerative colitis

2.0.2. Pathogenesis

Ulcerative colitis (UC) is a chronic disease characterized by diffuse mucosal inflammation limited to the large intestine. The disease usually begins in the rectosigmoid and may extend proximally in a symmetrical, circumferential, and continuous pattern to involve parts or the entire large intestine (Cotran et al., 1999). Strong evidence suggests that UC results from an abnormal or exaggerated intestinal immune response against stimuli that have not yet been identified. Pathologic changes begin with degeneration of the reticulin fibers beneath the mucosal epithelium, occlusion of the subepithelial capillaries, and progressive infiltration of the lamina propria with neutrophils, plasma cells, lymphocytes, eosinophils, mast cells, and macrophages (Carpenter and Talley, 2000; Macdermott and Stenson, 1988; Roediger, 1988). These cells secrete a variety of products known as inflammatory mediators, including cytokines, eicosanoids as well as oxygen and nitrogen metabolites (Macdermott and Stenson, 1988). Since inflammatory mediators can amplify local inflammation and directly cause tissue damage, it has been proposed that they are largely responsible for the clinical and histological changes seen in UC. Furthermore, as a consequence of duration and intensity of the inflammatory process, mucosal damage might develop into dysplastic lesions increasing the risk of UC-associated carcinoma. Some of the inflammatory mediators, demonstrated to be involved in the pathogenesis of the disease, will be discussed later in more detail.

Even though UC inflamed mucosa contains neutrophils (characteristic of acute inflammation) as one of the most prominent infiltrating cells, ulcerative colitis has been

viewed as a chronic disorder because of its prolonged clinical course and a histological picture that has predominant characteristics of chronic inflammation (Asakura, 1999; Macdermott and Stenson, 1988; Wallace, 1998). The presence of neutrophils can be explained by acute episodic attacks that become superimposed on chronic disease. In fact in 90% of cases, UC has a course of spontaneous exacerbations and remissions and less commonly an unremitting course or a course of only a single attack (Cooper et al., 2000; Tibble et al., 2000; Langholz et al., 1994). It has been estimated that at any point in time, 50% of UC patients are asymptomatic (remission stage can vary in length from months to years), 30% have mild symptoms and 20% have moderate to severe symptoms (exacerbation stage) (Ghosh et al., 2000; Langholz et al., 1994).

The most common symptom is mucoid diarrhea (with or without blood) accompanied with the abdominal pain and prominent feeling of rectal urgency. In more severe cases patients also might experience fatigue, fever, weight loss, as well as various others extracolonic problems (Ghosh et al., 2000; Schmehl et al., 2000; Cotran et al., 1999; Andreoli, 1997). The symptoms may come on quite slowly, over several weeks or months, or may start suddenly. Depending on the severity of the condition, symptoms may be very disabling for patients affecting the quality of their social life, education and working abilities (Lakatos et al., 2004; Nielsen et al., 2000).

2.0.3. Histopathology of ulcerative colitis

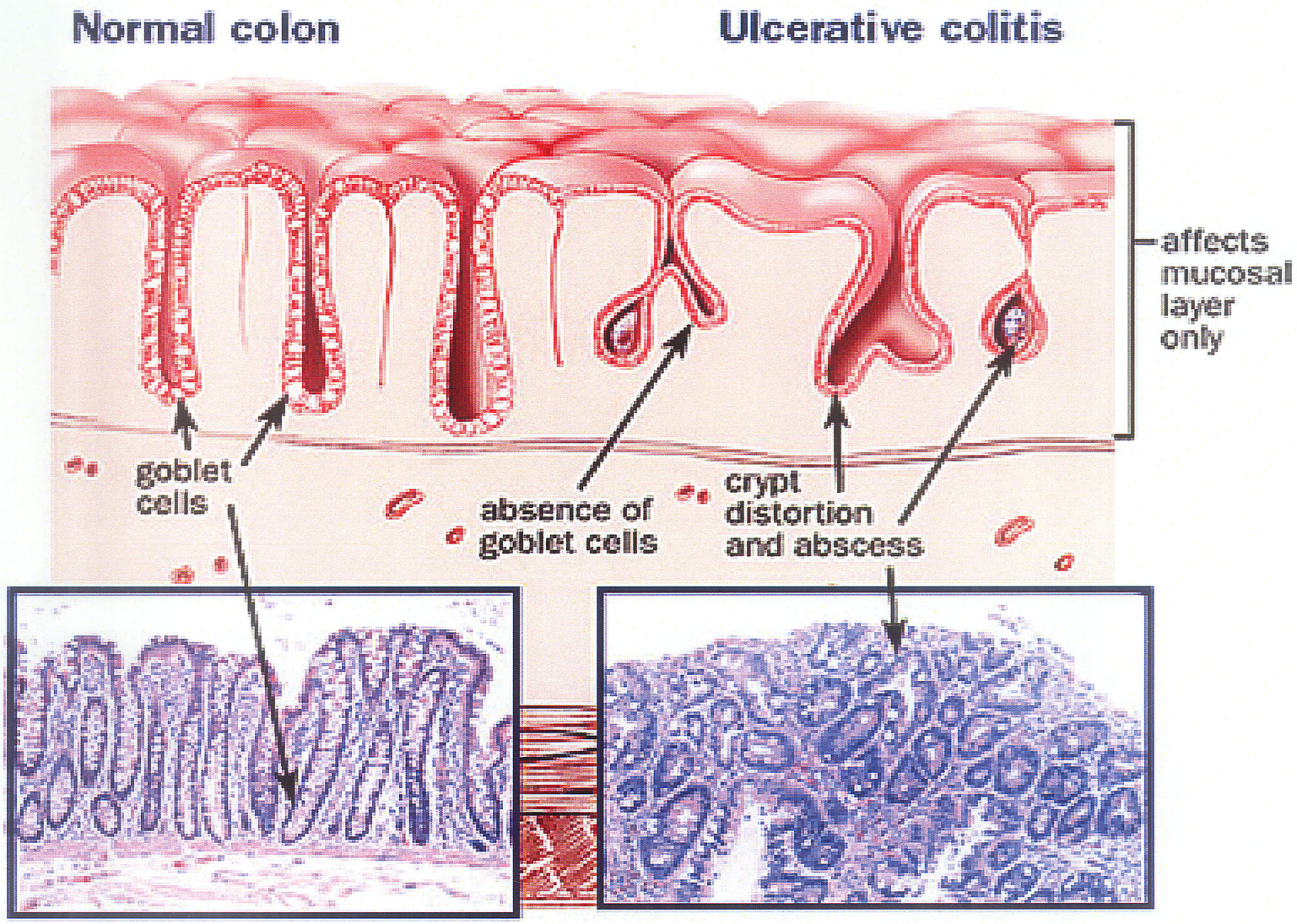
Since colonic mucosa can react to injury in only a limited number of ways, histological changes in UC are not disease-specific, but they rather reflect extent of injury and duration of disease. The histological pattern observed in severe/moderately severe

UC is characterized by diffuse mucosal inflammatory changes with congested lamina propria infiltrated by acute and chronic inflammatory cells (neutrophils, eosinophils, lymphocytes, plasma cells and mononuclears) (Cotran et al., 1999; Macdermott and Stenson, 1988). Vascularity is increased and submucosa width is either normal or reduced. The crypts show reduction of globet cells. Normal mucosal architecture is lost due to crypts abscess and distortion and branched regenerating crypts may form (Carpenter and Talley, 2000; Schmehl et al., 2000). Focal lymphoid hyperplasia can be observed in mucosa and superficial submucosa. Epithelial dysplasia might develop in multiple sites (Carpenter and Talley, 2000; Andreoli, 1997) (Figure 2.2.).

2.0.4. Treatment

Etiology of UC is unknown and there is no real cure for this disease. However, since all clinical symptoms appear to be direct consequence of the inflammatory process, the main goal of a treatment is to reduce inflammation during relapse and prolong the time spent in remission (Tibble et al., 2000). Various anti-inflammatory medications, immunosuppressive drugs, sedatives and antispasmodics are used to provide symptomatic relief (Kamm, 2002; Ghosh et al., 2000). In case of failure of medical therapy or dysplasticly changed colonic epithelium, colectomy is indicated. Up to 35% of UC patients undergo proctocolectomy (removal of rectum and colon) within the first 5 years of diagnosis (Ghosh et al., 2000; Tsianos, 2000; Langholz et al., 1994). This surgical procedure discontinues further episodes of UC and prevents development of UC-associated carcinoma.

Figure 2.2. Illustration of histological features as seen in normal human colonic mucosa and colonic mucosa affected by ulcerative colitis. (www.hopkins-gi.org)


Normal colon
Ulcerative colitis
affects mucosal layer only
goblet cells
absence of goblet cells
crypt distortion and abscess

2.1. Ulcerative colitis-associated colorectal cancer (UC-CRC)

2.1.0. Epidemiology

A substantial body of evidence supports the hypothesis that chronic inflammation may play a causative role in a variety of cancers. Some established examples of chronic inflammatory conditions known to heighten the risk of developing liver, stomach, or colorectal cancer are hepatitis, chronic *Helicobacter* infection, and IBD respectively (Shacter and Wietzman, 2002). The association between UC and colorectal cancer was first reported by Chron and Rosenberg in 1925, and then by Bergan in 1928. (Tsianos, 2000; Green et al., 1999). Since then, many authors have confirmed this association; however, the estimate of the risk of colorectal cancer among UC patients has varied widely from study to study. The clinically heterogenous nature of IBD, methodological problems and biases, including differences in patients' care as well as incomplete and too short follow-up (which often does not exclude patients with surgical removal of the colon), are probably among the most responsible factors influencing different incidence and prevalence reports, and disputes about the magnitude of UC-associated colorectal cancer risk (Tsianos, 2000; Karlen et al., 1999). Various studies have reported overall UC-related colorectal cancer incidence in a range from 3.7% to 13.5% and estimated that UC patients face a two- to sevenfold increased risk for colorectal cancer development compared to the normal population (Shacter and Wietzman, 2002; Eaden et al., 2001; Pohl et al., 2000). According to Eaden and Mayberry (2000), some recent population-based studies have reported that the incidence of UC-CRC is decreasing. Reasons for this are unclear but may include better diagnostic and treatment's options for UC patients as

well as better surveillance programs including early detection of mucosal dysplasia and subsequent surgical management (Hookman and Barkin, 2002).

2.1.1. Etiology

There are several independent risk factors thought to be important in the development of UC-related colorectal cancer, including extent of disease (primarily pancolitis), duration of disease (>8-10 years) and young age at onset (less than fifteen) are most frequently reported (Rhodes and Campbell, 2002; Eaden and Mayberry, 2000; Greenstein, 2000; Kornfeld et al., 1997; Choi and Zelig, 1994). The cumulative risk of colorectal cancer in UC patients has been estimated as about 7% at 20 years of disease, 14% at 25 years, and 30% and 40% after 35 years of disease for patients with pancolitis at diagnosis and for those who were diagnosed with UC before the age of 15 respectively (Eaden et al., 2001; Cooper et al., 2000; Tsianos, 2000; Bernstein et al., 1994). Other factors such as low activity of disease, geographical variation, primary sclerosing cholangitis (chronic liver disease that damages biliary tract develops in 2-5% of patients with UC (Brentnall et al., 1996), and family history of sporadic colon cancer have also been indicated (Rhodes and Campbell, 2002; Eaden and Mayberry, 2000; Ghosh et al., 2000; Greenstein, 2000; Tsianos, 2000). Furthermore, recent studies reveal that UC patients without pharmacological treatment have an increased risk for cancer development compared with patients treated with anti-inflammatory drugs (Eaden and Mayberry 2000; Tsianos, 2000; Kornfeld et al., 1997). This finding points to an important, although not a new theory (Virchow first proposed it in1863), that one or more

inflammatory components might have a significant role in the process of malignant transformation (Rhodes and Campbell, 2002).

2.1.2. Pathogenesis of UC-associated colorectal cancer

Inflammation is a complex stereotypical response of the body whose ultimate goal is to rid the organism of both the initial cause of cell injury and the consequence of such injury (Cotran et al., 1999). However, even though inflammation is fundamentally a protective reaction, inflammatory mediators if accumulated in large number have the potential to cause harmful effects. Lymphocytes, neutrophiles, eosinophils and macrophages, but also non-immune cells in the intestine, such as epithelial, muscle and nerve cells, are all found to be involved in inflammatory process. When activated by an inflammatory stimulus these cells produce inflammatory mediators that either can aggravate or suppress inflammation (Cotran et al., 1999). Inflammatory mediators, is a collective term for a group of different types of molecules, such as cytokines (e.g. TNF-alpha), reactive oxygen and nitrogen metabolites (e.g. nitric oxide), and eicosanoids (e.g. prostaglandins and leukotreins) (Macdermott and Stenson, 1988). Whether inflammation persists or whether it is eliminated depends on the balance between these molecules. Within colonic mucosa if pro-inflammatory mediators cause tissue damage and anti-inflammatory mediators are unable to restore balance and promote healing, the symptoms of UC will develop (De Dombal et al., 1993). Studies exploring inflammation-associated development of skin (Slaga et al., 1978), colon (Choi and Zelig, 1994), and bronchogenic carcinoma (Park and Aust, 1998) demonstrated that tumors generally develop at the sites

of the inflammation and not in surrounding non-affected tissue, suggesting the involvement of local inflammatory mediators in the process of malignant transformation.

The mechanisms underlying the frequent malignant transformation in UC patients with long-standing disease are not fully understood. A widely accepted theory is that chronic exposure to various inflammatory mediators might contribute to cancer development through their role in angiogenesis, mutagenesis, oncogene activation, increasing cell proliferation, and resistance to apoptosis (Rhodes and Campbell, 2002; Shacter and Wietzman, 2002; Cotran et al., 1999). The remainder of this chapter will focus on the interaction of three inflammatory molecules: TNF-α (tumor necrosis factor-alpha), iNOS (inducible nitric oxide synthase), and COX-2 (cyclooxygenase), each capable of contributing to the process of malignant transformation.

2.1.2.0. Preneoplastic inflammatory mediators

Pathological changes generated in affected tissue during long-lasting UC indicate that colitis-associated cancer develops secondary to the chronic inflammatory process. However, how malignant transformations develop from inflamed tissue and why adenocarcinomas develop in some patients and not in others remains a mystery. Efforts made to investigate mechanisms of injuries caused by chronic inflammation or cancer development resulted in emergence of molecules that are involved in both biological processes. Different molecular pathways have been proposed, and one of them hypothesized that the interaction between inflammatory molecules TNF-α, iNOS, and COX-2 is capable of generating, in words of Schwartsburd (2003), a potentially "vicious

self-sustaining loop(s)'" which results in a pro-cancer microenvironment favourable for malignant transformation, survival of tumor cells and their growth.

2.1.2.0.0. Tumor necrosis factor alpha (TNF-α)

TNF-α is one of the most potent pro-inflammatory cytokines. Cytokines are short acting, low molecular-weight peptides, active at very low concentrations, which are produced by inflammatory cells (mainly lymphocytes and macrophages), but also epithelium, endothelium and connective tissue cells, in response to pro-inflammatory stimulus, such as bacterial products, immune complexes, toxins, physical injury and other cytokines. High levels of pro-inflammatory cytokines, such as interleukin-1β, interleukin-6, interleukin-8, and TNF-α, in the colonic mucosa are found to play a prominent role in the pathogenesis of ulcerative colitis (Tian et al., 2003). These cytokines stimulate the immune system causing an inflammatory reaction, which can produce tissue damage in the intestinal mucosa.

The role of TNF-α in mediation of inflammatory process can be done directly by chemoattracting neutrophils and monocytes, activating helper T cells, eosinophils and mast cells, inducing endothelial adhesion molecules, and increasing microvascular permeability (Bischoff et al., 1999; Cotran et al., 1999), or indirectly by inducing the production of other pro-inflammatory cytokines, growth factors, and pro-inflammatory enzymes and their products such as iNOS (i.e. nitric oxide) and COX-2 (i.e. eicosanoids) (Tian et al., 2003; Cotran et al., 1999) (Figure 2.3.).

The concentration and corresponding TNF-α mRNA expression levels are found to be significantly elevated in chronically inflamed colonic mucosa (Colon et al., 2001;

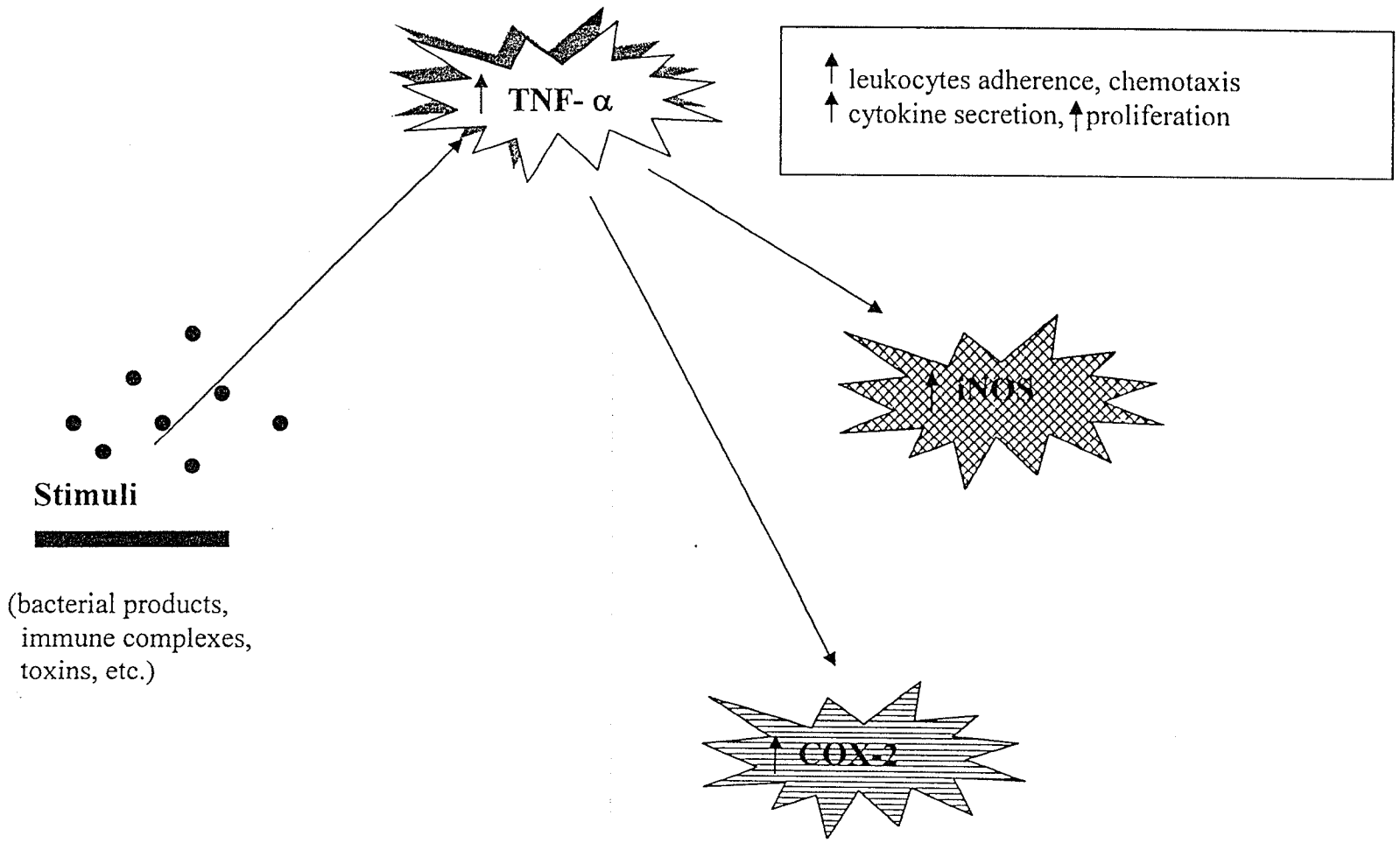
Woywodt et al., 1999). The involvement of TNF-α in pathophysiology of UC is supported by a study done by Neurath and colleagues (1997), which showed that mice deficient in TNF-α do not develop chronic colitis. In addition, clinical trials reported ameliorated clinical symptoms in UC patients treated with specific TNF-α inhibitors (Su et al., 2002; Evans et al., 1997).

The role of TNF-α in tumorigenesis has been demonstrated in several studies. Tumor cells, developing under inflammatory conditions, are found to gain a growth advantage by acquiring the ability to proliferate in response to TNF-α (Shacter and Weitzman, 2002). By inducing the nuclear factor kappa B (NF-κB, an ubiquitous transcription factor found to play a critical role in the cellular response to TNF-α 's pro-inflammatory signal), TNF-α was shown to inhibit apoptosis in human malignant epithelial cells (Van Antwerp et al., 1996). Furthermore, TNF-α contributes to progression of both, inflammation and carcinogenesis, by up-regulating inducible enzymes iNOS and COX-2 involved in the regulation of several biologic processes responsible for tissue damage and tumor growth.

2.1.2.0.1. Inducible nitric oxide synthase (iNOS)

Research showed that increase in TNF-α-release triggers expression of inducible nitric oxide synthase (iNOS or NOS-2) (Marion, et al., 2003; Colon et al., 2001). iNOS belongs to a family of nitric oxide synthases (NOSs) which catalyze the oxidative deamination of L-arginine into L-citruline producing nitric oxide (NO), a reactive nitrogen gas involved in a wide range of physiological and pathophysiological

Figure 2.3. Proposed role of TNF-α in the inflammatory process and associated carcinogenesis by direct involvement and indirectly by up-regulation of iNOS and COX-2.


TNF- α
↑ leukocytes adherence, chemotaxis
↑ cytokine secretion, ↑proliferation
↑ iNOS
Stimuli
(bacterial products,
immune complexes,
toxins, etc.)
↑ COX-2

actions (Lala and Chakraborty, 2001).

Three isoforms of NOSs have been identified. Two constitutively expressed calcium-dependent, endothelial and neuronal (eNOS and nNOS), which produce a small amount of NO due to transitory increases in intracellular calcium, and a third, iNOS, an independent isoform which when expressed in response to pro-inflammatory agents, such as endotoxins and cytokines, continuously generate substantial amounts of NO for as long as pro-inflammatory stimuli is present (Alderton et al., 2001). NO is synthesized by iNOS in various cell types including macrophages, neutrophils, and certain carcinoma cells.

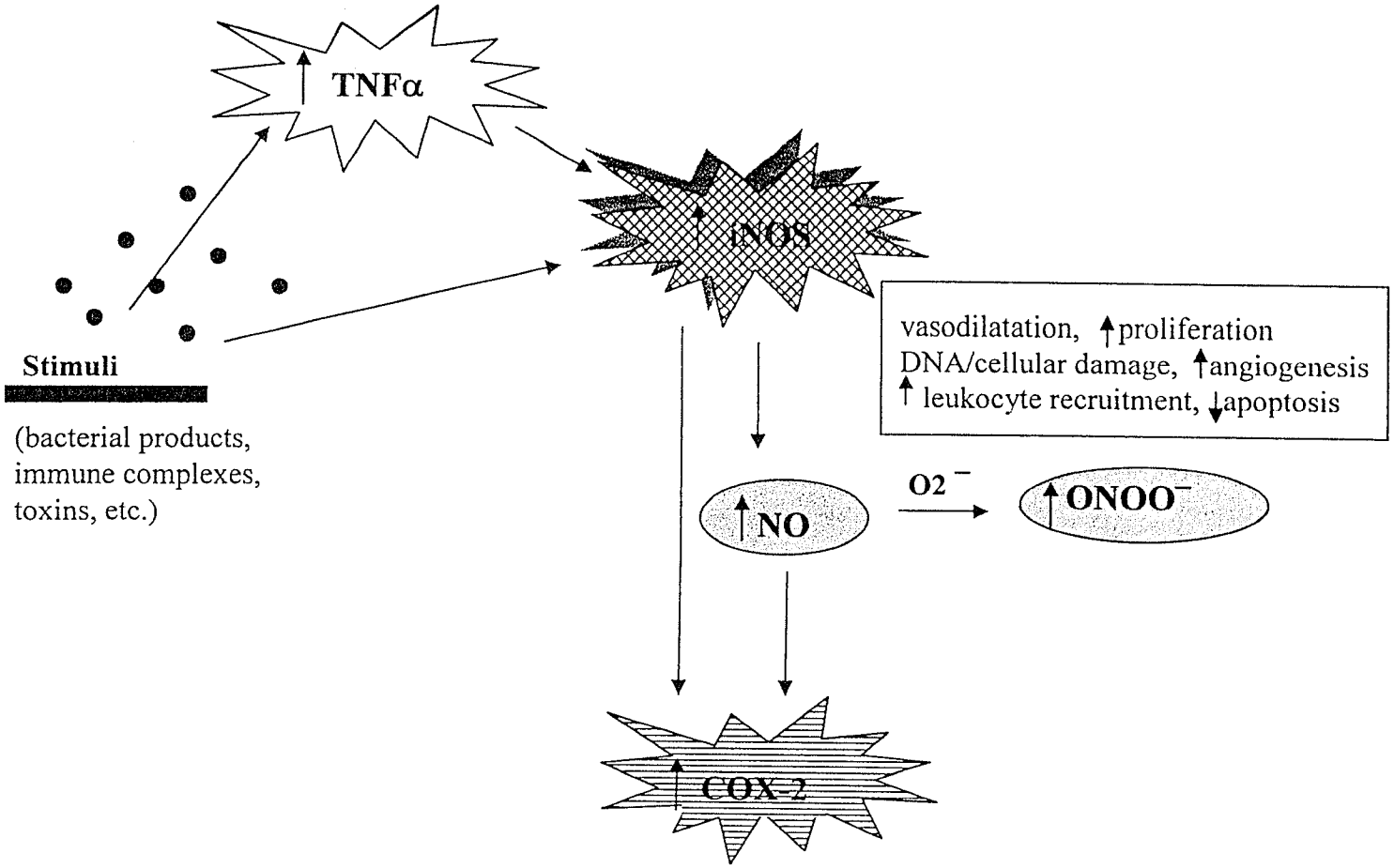
In living organisms, NO can react with O_2 to form nitrogen dioxide (NO_2-) or with superoxide anions (O_2-), to form highly cytotoxic peroxynitrite radicals (ONOO-) (Dijkstra et al., 1998). Under physiological circumstances NO has the protective role, as seen in the regulation of integrity and repairing of the gastric mucosa, and protection against different aggressive agents (Calatayud et al., 2001). However, when over-expressed NO and its metabolites can have very damaging effects. Peroxynitrite, for example, induces nitration of tyrosine residues on proteins (nitrotyrosine), leading to changes of protein structure and function. Also, via oxidative mechanisms peroxynitrite can damage DNA by causing DNA base modifications and strand breaks (Alderton et al., 2001; Kennedy et al., 1998). There is a general agreement that nitrosatively and oxidatively modified proteins and DNA, identified in chronically inflamed tissues, contribute to gene mutations and development of carcinoma.

Besides acting as an initiator of carcinogenesis, NO can be involved in tumor promotion by up-regulating angiogenesis via stimulation of vascular endothelial growth

factor (VEGF) (Chin et al., 1997). Other actions stimulated by iNOS and subsequently generated reactive nitrogen oxide species, are activation of nuclear transcription factors, up-regulation of COX-2, modulation of cytokines, recruitment of leukocytes, induction or inhibition of apoptosis and increased metastatic potential (Payne et al., 1999; Lala and Chakraborty, 2001; Abramson et al., 2001) (Figure 2.4.). Nevertheless, some studies have reported anti-tumor properties of induced NO (Liu et al., 2003; Xu et al., 2002).

Elevated levels of iNOS as well as nitric oxide (NO) have been found in inflamed mucosa of patients with UC (Kankuri et al., 2003; Dijkstra et al., 1998). iNOS over-expression has also been demonstrated in several animal models of IBD (Colon et al., 2001; Southey et al., 1997). Different studies even on the same type of tumors reported different results regarding levels of iNOS expression. While some authors reported an increase (Takahashi et al., 1997; Rao, et al., 1998; Kojima et al., 1999;) others (Moochhala et al., 1996; Hao et al., 2001) reported decreased iNOS mRNA, protein and/or NO levels in ACF and carcinoma tissues, compared with the levels found in normal colonic epithelium. These controversial reports on the effects of iNOS and its products seem to depend on many factors including balance with other inflammatory molecules, the timing, and concentration of NO. Low concentrations of NO are thought to induce angiogenesis and stimulate cell growth, whereas high concentrations are described as cytotoxic and able to induce apoptosis (Liu et al., 2003).

Figure 2.4. Proposed role of iNOS in the inflammatory process and inflammation-associated carcinogenesis by synthesis of reactive nitrogen species and by up-regulation of COX-2.


TNFα
iNOS
Stimuli
(bacterial products,
immune complexes,
toxins, etc.)
vasodilatation, ↑proliferation
DNA/cellular damage, ↑angiogenesis
↑ leukocyte recruitment, ↓apoptosis
↑NO
O2 −
↑ONOO−
↑COX-2

Although the precise role of iNOS in inflammation and carcinogenesis is controversial, its inhibition has been effective in amelioration of colitis-associated tissue damage (Kankuri et al., 2001), and reduction of ACF formation (Rao et al., 1999). One important ability of iNOS and its products, demonstrated in variety of experimental models, is to enhance inflammatory process via up-regulation of expression and activity of COX-2. Rao and colleagues (1999), showed that a specific iNOS inhibitor when administrated to AOM-treated rats significantly reduced ACF formation and suppressed carcinogen-induced iNOS and COX-2 activities in colonic mucosa. In work on precondition ishemic myocardium, it was demonstrated that COX-2 elevation was dependent on generation of NO by iNOS, indicating that COX-2 was downstream of iNOS (Shinmura et al., 2002). Nogawa and colleagues in 1998, showed that NO produced by iNOS increases COX-2 activity after cerebral ischemia, while Van Hoogmoed et al., (2002), demonstrated that there is a correlation between release of NO and the production of prostaglandins in the smooth muscle of the large colon. When the induced forms of both enzymes, iNOS and COX-2, are over-activated during an inflammatory insult they contribute to amplified tissue injury and may act as an important regulatory mechanism in malignant transformation of cells injured by inflammation.

2.1.2.0.2. Cyclooxygenase-2 (COX-2)

The function of cyclooxygenase (COX) is to convert arachidonic acid (AA) to inflammatory and other physiological mediators such as prostaglandins and thromboxane. Two isoforms of COX are identified: COX-1 is constitutively expressed in most tissues and involved in homeostatic functions such as regulation of renal blood flow, platelet

aggregation, and maintaining of normal gastric mucosa, whereas COX-2 is the inducible form, expressed at sites of inflammation, involved in the production of lipid inflammatory mediators implicated in pain and inflammation (Simon, 1999). The main lipid inflammatory mediators observed in inflamed intestinal tissue are prostaglandin E2 (PGE2), prostaglandin I2 (PGI2) and thromboxane A2 (TxA2) (Schumert et al., 1988). These pro-inflammatory lipid molecules are also known as eicosainoids (a group term for all metabolites of AA). COX-2 is synthesized in monocytes and macrophages, but also in fibroblasts, epithelial and endothelial cells (Singer et al., 1998) after exposure to pro-inflammatory agents including reactive nitrogen species, bacterial cell products, inflammatory cytokines and tumor promoters (Surh et al., 2001). As previously (2.1.3.0.0.) mentioned TNF-α involvement in the inflammatory process is in part due to its ability to enhance COX-2 activity. Experiments on osteoblastic and colonic epithelial cell lines showed elevation of COX-2 mRNA and protein levels after exposure to TNF-α; this elevation was explained by TNF-α's ability to up-regulate NF-κB, which in turn stimulates expression of the COX-2 gene (Jobin et al., 1998; Yamamoto et al., 1995). The involvement of iNOS and its metabolites in the enhancement of COX-2 expression was reviewed in the previous paragraph (2.1.3.0.1).

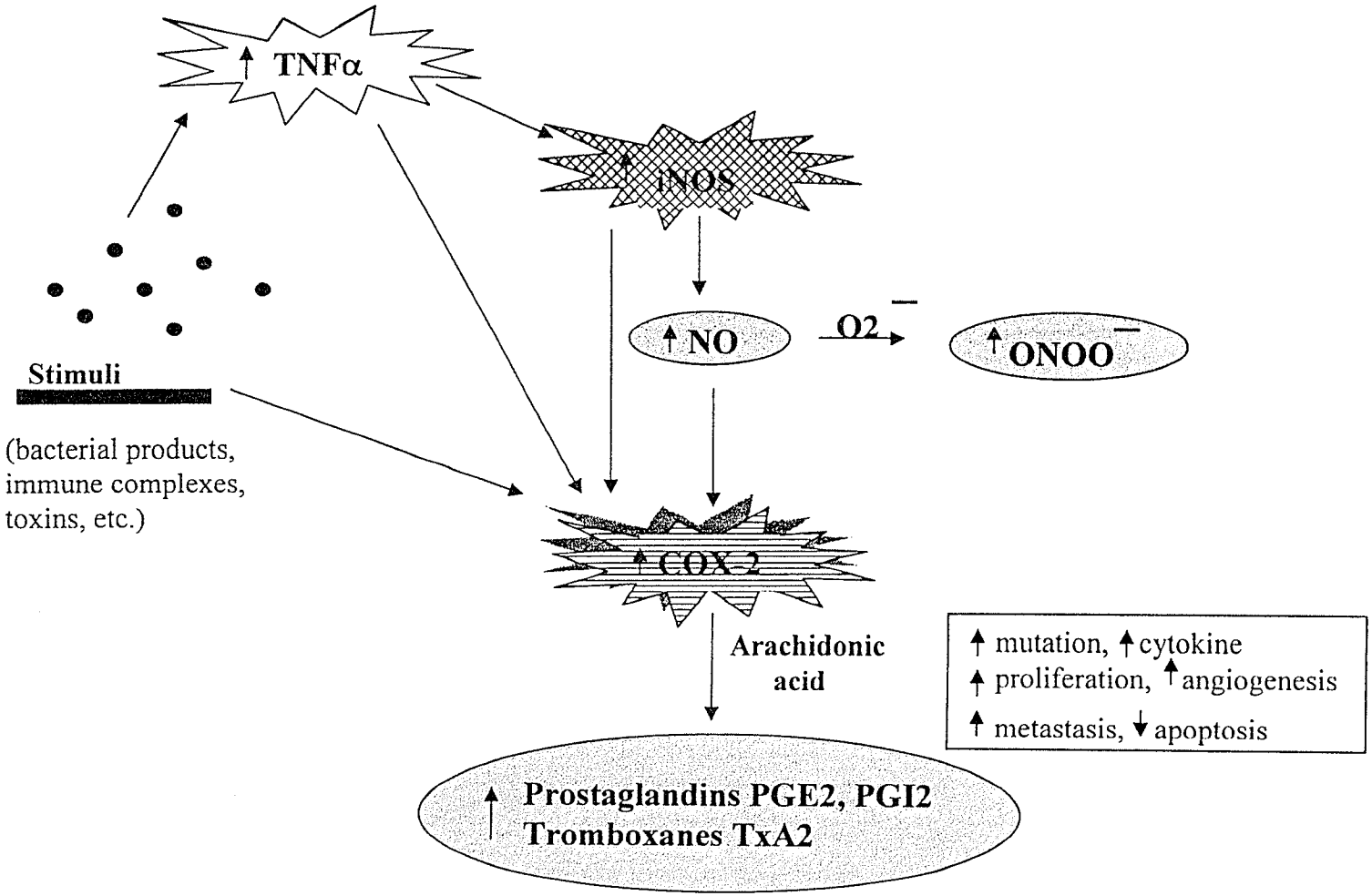
There is increasing evidence that COX-2 plays a major role in mediation of both pathological processes: inflammation and carcinogenesis. Human and animal studies have demonstrated over-expression of COX-2 and its products (PGE2, PGI2 and TxA2) in UC (Singer et al., 1998; Reuter et al., 1996; Raab et al.,1995; de Dombal et al., 1993) as well as in colonic adenomas and carcinomas (Shacter and Weitzman, 2002; Shattuck-Brandt et al., 2000; Kawamori et al., 1998; DuBois, et al., 1996; Eberhart et al., 1994). It

has been shown that the expression of COX-2 and lipid mediators of inflammation corresponds to UC activity and increases during multistage tumor development, indicating that there is a link between COX-2 activity, inflammation and tumor growth (Hendel and Nielsen, 1997). A recent study by Hill and colleagues (2004), is one of the rare investigations performed on the involvement of COX-2 in UC-CRC development. The authors compared carcinomas arising in inflammatory bowel disease versus sporadic carcinomas and found significantly increased expression of COX-2 in colitis-associated carcinomas.

It is not clear how COX-2 promotes colorectal tumorigenesis. Some researchers explain it by COX-2's role in production of several eicosanoids that are involved in regulation of cell growth and differentiation. Eicosanoids, especially PGE2, are known to modulate various biological processes favourable to cancer development, including mutation, stimulation of cell proliferation, induction of cytokines synthesis, induction of angiogenesis, immunosuppression, promotion of metastasis, and inhibition of apoptosis (Harris et al., 2002; Prescott and Fitzpatrick, 2000) (Figure 2.5). On the other hand, since a high concentration of free AA is shown to advance apoptosis, prostaglandin independent mechanism by which COX-2 may promote tumorigenesis was proposed. This proposal is based on COX-2 ability to deplete intracellular levels of free AA and thereby inhibit apoptotic process (Prescott and Fitzpatrick, 2000).

A large number of epidemiological studies have observed that regular, long-term use of non-steroidal anti-inflammatory drugs (NSAIDs - known to inhibit cyclooxygenase activity and subsequent prostaglandin synthesis) decreases the risk of colorectal cancer by 40% to 50% (Herendeen and Lindley, 2003; Levy, 1997;

Figure 2.5. Proposed role of COX-2 in the inflammatory process and inflammation-associated carcinogenesis by synthesis of pro-inflammatory prostaglandins (PGE2, PGI2) and thromboxane (TxA2).


TNFα
iNOS
NO
O2 −
ONOO −
Stimuli
(bacterial products,
immune complexes,
toxins, etc.)
COX-2
Arachidonic
acid
↑ mutation, ↑ cytokine
↑ proliferation, ↑ angiogenesis
↑ metastasis, ↓ apoptosis
↑ Prostaglandins PGE2, PGI2
Tromboxanes TxA2

Giovannucci et al., 1995; Thun et al., 1993). NSAIDs, including aspirin, sulindac, and indomethacin, were then experimentally tested and their anti-carcinogenic ability was confirmed in a variety of chemically induced tumors (Reddy et al., 1993). In patients with familial adenomatous polyposis, NSAIDs were also proved beneficial by decreasing the number and size of adenomas (Giardiello et al., 1993). Similar anti-cancer effects of NSAIDs were demonstrated in randomized controlled trial on patients with history of colorectal cancer whose daily use of aspirin for 31 months reduced the incidence of new adenomas (Sandler, et al., 2003). However, it was soon discovered that since traditional NSAIDs non-selectively inhibit activity of both COX enzymes, administration of these drugs might result in unwanted gastrointestinal side effects caused by inhibition of beneficial COX-1 activity. This downside of NSAIDs urged the need to develop selective COX-2 inhibitors, which would keep the anti-cancer abilities of NSAIDs but lower occurrence of side effects caused by inhibition of COX-1. As expected, administration of selective COX-2 inhibitors showed lower prevalence of side effects, compared to non-selective NSAIDs (Ukawa et al., 1998). Moreover, as shown in various studies, COX-2 inhibitors decreased both, the frequency and number of premalignant and malignant lesions, in rodent models of colorectal carcinogenesis (Rao et al., 1999; Kawamori et al., 1998), inhibited growth of human colonic cancer cell lines (Sheng et al., 1997), as well as reduced the mean number of polyps in patients with familial adenomatous polyposis (Clapper et al., 2001). These findings were further proof for "exclusive" involvement of COX-2 and its metabolic pathway in the pathogenesis of inflammation and colorectal cancer development.

There are controversial reports on the use of NSAIDs and selective COX-2 inhibitors in animal models of experimentally induced colitis. Karmeli and his group (2000), reported that COX-2 inhibition ameliorates the severity of inflammation in experimentally induced-colitis, while Reuter and colleagues (1996), reported exacerbation with colonic perforation after administration of COX-2 selective inhibitors to rats with chemically induced colitis. However, a retrospective cohort study, which included 1059 UC and 881 CD patients followed for 3 years, reported that use of NSAIDs proved beneficial in pain management and was not associated with disease flares (Agoff et al., 2000). Furthermore, a recently published study by Takeda and colleagues (2004), demonstrated that treatment with a COX-2 selective inhibitor, etodolac, suppressed development of aberrant crypt foci and tumors in a colitis-associated tumorigenesis model. Even though promising in inhibition of sporadic colon cancer development, further investigation is needed to clarify the role of COX-2 inhibitors in ulcerative colitis and colitis-associated carcinogenesis.

2.1.3. Possible role of inflammation in pathogenesis of sporadic colorectal cancer

Since colon and rectum are repeatedly exposed to the various pro-inflammatory agents, (e.g. chemical irritants, non-digestible particles, bacterial, viral, and parasitic infections), it is speculated that "normal" colorectal mucosa is in a continual state of low-grade inflammation. That is why some authors have proposed that chronic inflammation, as a common underlying process, may be important in the pathogenesis of both UC-

associated as well as sporadic colorectal cancer (Rhodes and Campbell, 2002). This theory is supported by many similarities observed between sporadic and UC-associated cancers. For example, both cancers evolve from pre-cancerous dysplastic mucosa through a multistep progression pathway; the genetic alterations found in cancer complicating UC involve many of the same targets found in sporadic cancer; both lack clinical symptoms during early stages of development and are characterized by obstructive symptoms, rapid weight loss, and abdominal masses in advanced stages; and lastly the prognosis for both UC-associated cancer and its sporadic counterpart is similar (Rhodes and Campbell, 2002; Eaden and Mayberry, 2000; Greenstein, 2000; Cotran et al., 1999; Fracasso et al., 1999). Moreover, as reviewed previously, inflammatory mediators TNF, iNOS and COX-2 and their products are over-expressed in sporadic carcinomas and their inhibition leads to a decrease in frequency and number of premalignant and malignant lesions. Therefore, investigation of UC-CRC might as well provide important clues about pathogenesis of sporadic colorectal cancer.

2.2. Summary

A substantial body of evidence supports the hypothesis that chronic inflammation plays a causative role in colitis-associated carcinogenesis. The mechanisms underlying the frequent malignant transformation in UC patients, however, are not fully understood. The most often reported independent risk factor for development of colon cancer in UC patients is a long-lasting disease (> 8 years). These reports are based only on epidemiological observations and to our knowledge no experiments have been performed

to investigate the risk of colorectal cancer development as affected by duration of inflammation.

Different molecular pathways have been proposed in the development of colitis-associated carcinogenesis. TNF-α- iNOS-COX-2 pathway is particularly interesting because the pathological changes generated in colitis-affected tissue via this pathway might be the one responsible for gene mutations and development of carcinoma. Many human and animal studies found elevated levels of these important mediators of inflammatory process and their products in colitis-affected mucosa as well as in sporadic colon adenomas and carcinomas. The involvement of these molecules to initiate and promote tumor growth (including mutation, proliferation, and neovascularization) has also been confirmed. However, most of the research has employed either colitis-induced models or sporadic cancer models, and few studies have employed a UC-CRC model to address these questions. Usage of UC-CRC model is the most appropriate way to investigate specific events during the inflammatory process that could be responsible for initiation and/or promotion of inflammation-associated colon carcinogenesis. In other words, the knowledge obtained through such experiments would provide a better understanding of the timing of individual molecular and morphological events that predispose neoplastic transformations in colitis-affected colon. These findings then could be used for development of therapeutic methods designed to prevent malignant process in patients with ulcerative colitis.

2.3. Animal models of UC-CRC

Animal models provide a unique opportunity to investigate the role of

inflammation on development of colon cancer under strictly controlled laboratory conditions. They allow study of multistage events in ways not possible in humans, thus complementing and extending clinical investigations and adding to the understanding of etiology and pathogenesis of the human disease.

To be able to study the influence of inflammation on colorectal carcinogenesis, it is important to have a model that, ideally, by spontaneous malignant transformation or after introduction of carcinogen allows investigation of cellular and molecular events associated with cancer progression under inflammatory conditions. Various spontaneous, genetic and chemically induced models of colitis have been employed recently to study the process of colitis-associated carcinogenesis (Dieleman et al., 1997; Elson et al., 1995; Kim and Berstad, 1992; Strober, 1985). Even though none of these models resembles human disease completely, each provides certain similarities to the disease as noted in man, and can therefore contribute to our understanding of its nature.

The highest degree of similarity to human disease is observed in the cotton-top tamarin model. This endangered species of Colombian monkeys, not widely available for laboratory research, develops spontaneous colitis and subsequent colon adenocarcinoma when in captivity (Elson et al., 1995; Kim and Berstad, 1992). Some genetically engineered models such as interleukin 10 knockout and Gα 1-2 knockout mice have also been shown to spontaneously develop colitis-associated carcinoma (Berg et al., 1995; Rudolph et al., 1995). These models, however, are not the most appropriate to study human UC-CRC because they are genetically modified and therefore do not really replicate the pathophysiology of the human disease. The most widely used models of IBD are those in which the disease is induced experimentally by oral or rectal administration

of exogenous agents including: toxic chemicals (e.g. acetic acid, formalin, dextran sulphate sodium, or carrageenan); tissue homogenates from patients with IBD; infection agents; or by immunological manipulation (Dieleman et al., 1997; Elson et al., 1995; Kim and Berstad, 1992; Strober, 1985). One of the best-studied models of a chemical induction of ulcerative colitis is degraded carrageenan-induced model.

2.3.0. Carrageenan (CAR)-induced colitis

Carrageenan (CAR) is a sulfated polysaccharide obtained commercially by the aqueous extraction of red seaweeds (*Rhodophyceae*). Due to its ability to substitute for fat and combine easily with milk proteins, CAR is widely used in the food industry as a thickening, gelling and stabilizing agent. κ, λ, and ι are three most common forms of CAR. The difference among them is in degree of sulfation, and ability to form gels under different conditions. As a food additive CAR is allowed in concentrations of up to 2% in various food items including milk, pudding, yogurt, jams, processed low-fat meats, bakery products and even infant formulas (Tobacman, 2001; Watanabe et al., 1978). Food grade, or undegraded CAR, has a molecular weight of 100,000-800,000. Watt and Marcus were among the first researchers to report that when degraded by acid hydrolysis to lower molecular weights (20,000-40,000) the oral administration of CAR creates colonic lesions in various laboratory animals including guinea pigs, rats, mice, rabbits and monkeys (Tobacman, 2001; Watanabe et al., 1978; Ishioka et al., 1987). Recently, it has been shown that an antibiotic (metronidazol) could prevent CAR-induced colitis if given to the guinea pigs early in the course of CAR administration. This finding

suggested that anaerobic bacteria, sensitive to metronidazol, such as *Bacteroides vulgatus* might play a role in the initial events of CAR-induced colitis (Dieleman, 1997; Kim and Berstad, 1992; Onderdonk, 1985).

However, the generally accepted explanation for the mechanism of CAR-induced inflammation involves uptake of CAR by intestinal macrophages, followed by leakage of lysosomal enzymes into the mucosal tissue and consequent tissue destruction and inflammation (Kim and Berstad, 1992; Strober, 1985; Fabian et al., 1973). PGE2 levels of colonic tissue are elevated early in CAR-induced colitis, suggesting that PGE2 production is involved in the inflammatory response (Kim and Berstad, 1992). Immunologic mechanisms are also proposed, since CAR is found to activate the complement system (Kim and Berstad, 1992). The histopathogenesis of CAR-induced colitis is similar to that seen in humans. Injury is confined to the mucosa. The onset of inflammation is slow, early lesions are characterized with focal infiltration of epithelium with neutrophils, while late lesions are characterized by loss of crypts, crypt distortion, necrosis of the crypt epithelium, lymphocytic infiltration, capillary congestion of the lamina propria and atypical epithelial hyperplasia (Dieleman et al., 1997; Pricolo et al., 1996; Elson et al., 1995; Onderdonk, 1985). Clinical signs include weight loss, mucous, visible and/or occult blood in stools, and diarrhea (Tobacman, 2001; Elson et al., 1995).

Even though degraded CAR is the form mostly used to induce colitis, studies that employed undegraded carrageenan have reported epithelial cell loss and erosions as well as the development of intestinal ulcerations in different animals (reviewed in Tobacman, 2001). In a recent study (Dahleh, 2001) it was shown that the use of undegraded CAR induces sustained inflammation without massive ulceration and bleeding. Diarrhea was

the only clinical sign. Histological examination of colonic mucosa demonstrated crypt abscesses, lymphocytes and macrophages infiltration, and increase in the size of Peyer's patches. The increased levels of the pro-inflammatory mediators iNOS and COX-2 were observed in colonic mucosal samples (Dahleh et al., 2001). Moreover, we also showed in collaboration with the Institute for Biodiagnostics (National Research Council of Canada, Winnipeg, Manitoba) by using magnetic resonance spectroscopy (MRS), that the colonic mucosal samples of Sprague-Dawley rats fed just for four days with undegraded (food grade) CAR (2% by weight) had significantly elevated levels of choline and taurine bands, and significantly reduced level of methylene band (Bezabeh et al., 2003). These results correlate with the elevated level of choline band found in the MRS spectrum of colonic mucosal samples from UC patients (Bezabeh et al., 2001).

CAR has been shown to promote a carcinogenic effect in animals treated with different carcinogen chemicals (Tobacman, 2001; Corpet et al., 1997; Watanabe et al., 1978). Furthermore, long-term treatments using degraded CAR have revealed that given in high doses over a long period of time CAR might act as carcinogen itself (Tobacman, 2001; Ishioka et al., 1987; Fabian et al. 1973). These findings raised debate about the safety of CAR as food additive. Some researchers even believe that there is a strong association between carrageenan usage in the food industry and increased incidence of colitis during the 20^{th} century (Tobacman, 2001).

In the present study we selected to use the undegraded CAR-induced colitis model because of a histopathological similarity between carrageenan-induced colitis and ulcerative colitis in humans; sustained inflammation without massive ulcerations and bleeding which allows additional application of carcinogen avoiding extreme injuries;

low level of discomfort for involved animals; simplicity of preparation and administration; and wide application of food grade (i.e. undegraded) CAR in the food industry.

CHAPTER 3

3.0. Dietary fat and colon cancer

Several epidemiological and case-control studies have demonstrated a positive association between consumption of fat, especially fat of animal origin, and increase in incidence of colon cancer (Giovannucci and Willett, 1994; Hursting et al., 1990; Willett et al., 1990; Potter and McMichael, 1986). In general, the effects of high levels of dietary lipids on chemically induced colon carcinogenesis has shown that diets high in animal fat (lard or beef tallow), corn oil and safflower oil are tumor enhancing, while diets high in fish oil, coconut oil, or olive oil had no colon tumor promoting effects compared to their respective low fat diets (reviewed in Reddy, 1992). These findings suggest that not just total amount but also the fatty acid (FA) composition of dietary lipids plays a role in colon carcinogenesis. Beef tallow and lard are rich sources of saturated fatty acids: palmitic (16:0) and stearic (18:0), corn oil and safflower oil are rich in linoleic (18:2 ω-6) polyunsaturated fatty acid (PUFA), fish oil is rich in long chain ω-3 PUFAs: eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3), olive oil is rich in monounsaturated oleic fatty acid (18:1 ω-9), and coconut oil is rich in medium chain lauric fatty acid (12:0) (Table 3.1.). The argument here is that the composition of ingested dietary fat is critical to colon cancer risk through ability of FAs to modulate cellular membrane functions. Different fatty acids possess different biological activities and it was shown that as many as 40 FAs can be incorporated into phospholipid molecules making up the plasma membrane (Clandinin et al., 1991). Therefore, the type and amount of FAs ingested are able to affect cellular

Table 3.1. Percentage of major fatty acids present in commonly used dietary lipids (%)

Fatty acid	Corn oil	Safflower oil	Olive oil	Beef tallow	Perilla oil	Flaxseed oil	Menhaden oil	Coconut oil
C 8:0								8.8
C 10:0								6.2
C 12: 0								**48.8**
C 14: 0				3.4			8.4	20.0
C 14: 1								
C 15: 0								
C 16: 0	11..5	9.5	9.6	**25.4**	8.3	9.0	15.2	7.8
C 16:1				3.6			11.6	
C 18: 0			2.7	**22.6**				3.0
C 18: 1ω9	22.6	12.3	**80.6**	38.2	14.3	18.0	9.5	4.0
C 18: 2ω6	**58.7**	**74.0**	5.8	4.0	15.3	16.0		
C 18: 3ω3					**58.2**	**57.0**		
C 20:5ω3							**16.0**	
C 22: 5ω3							4.0	
C 22: 6ω3							**10.8**	

The fatty acid composition of dietary lipids citied in Table 3.1. is obtained from following sources: (Reddy, 1992; Flax Council of Canada; Okuno et al., 1997; www.coconutoil-online.com)

responses via modification of the activity of membrane receptors and other signalling intermediates leading to altered gene expression (Clandinin et al., 1991).

Mechanism proposed to account for the tumor promoting ability of high fat beef tallow and high fat corn oil diet is in their ability to increase the concentration of secondary bile acids in colon which, in turn, induce cell proliferation and act as promoters in colon carcinogenesis (Bull et al., 1993). In addition, it has been shown that the high intake of these fats enhances colon tumor promotion by altering membrane phospholipid turnover, enhancing release of membrane AA, and thus increasing synthesis of pro-inflammatory prostaglandins (Reddy, 1992). On the other hand, the protective role of fish oil and perilla oil (rich in α-linolenic acid) have been associated with the ability of ω-3 PUFAs to suppress the biosynthesis and concentration of AA, and thus increasing synthesis of less pro-inflammatory mediators (Caughey et al., 1996; Narisawa et al., 1994). Moreover, it has been also demonstrated that fish oil supplementation limits production of secondary bile acids (Roynette et al., 2004).

3.1. ω-6 and ω-3 polyunsaturated fatty acid (PUFAs)

ω-6 and ω-3 fatty acids are structural components of membrane phospholipids and therefore are involved in many important roles including regulation of membrane fluidity, cellular signalling and cellular interaction (Roynette et al., 2004). The role of ω-6 and ω-3 fatty acids as precursors for eicosanoids synthesis has attracted the interest of many researchers in recent years. Eicosanoids are a group of 20-carbon fatty acids whose production begins with the liberation of AA or EPA from membrane phospholipids by

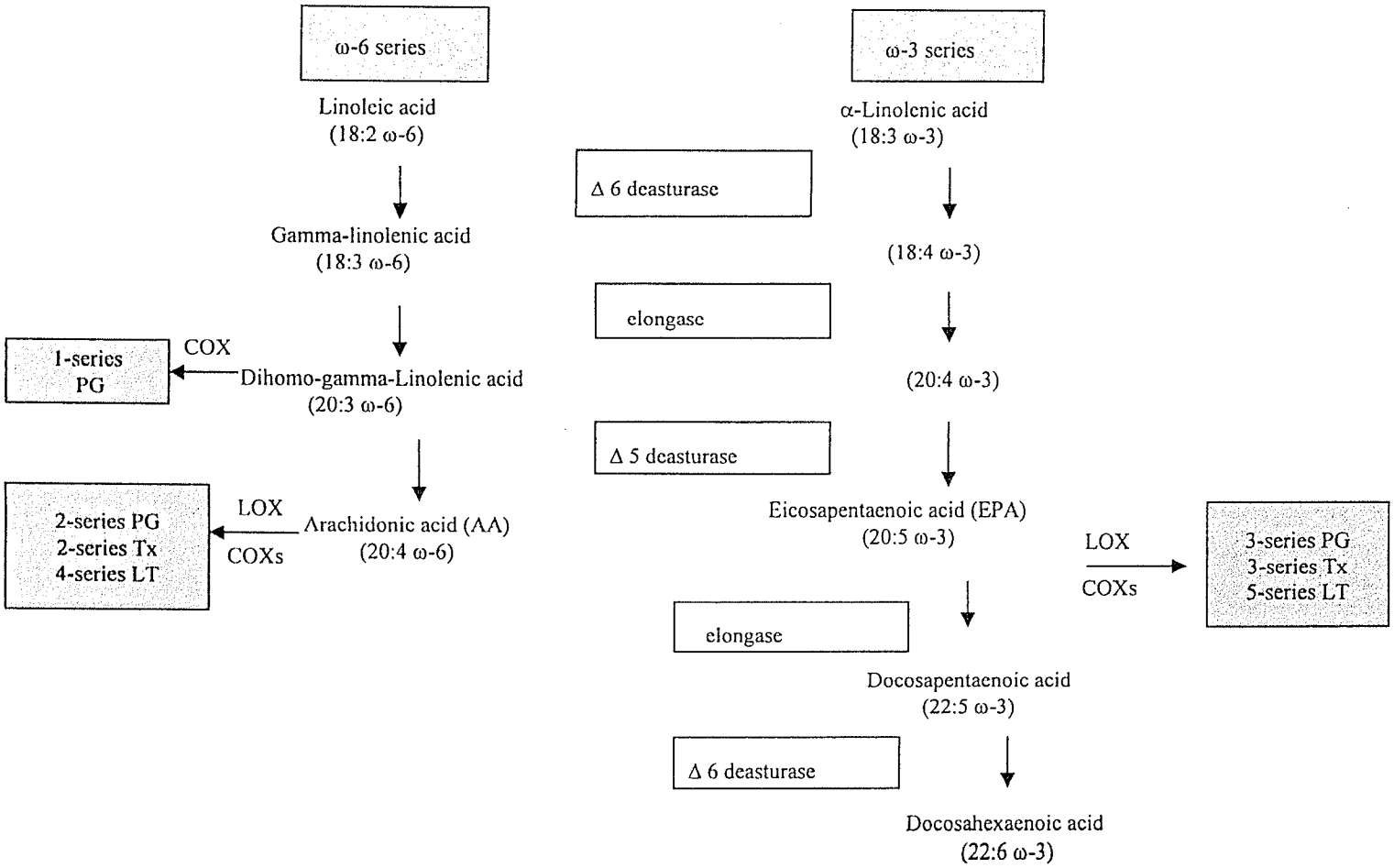
the action of phospholipase enzymes (especially phospholipase A2 and C) in response to inflammatory stimuli (Simon, 1999). Liberated AA or EPA is than metabolised by cyclooxygenase (COX) or lipoxygenase (LOX) enzymes. The synthesis of prostaglandins (PGs) and thromboxanes (TXs) is dependent on the action of COX, whereas the synthesis of leukotrienes (LTs) is dependent on the action of LOX. Because it normally predominates in cell membrane, AA is the principal precursor for eicosanoid synthesis and gives rise to PGE2, TXA2 and LTB4, which are known potent pro-inflammatory mediators also shown to promote carcinogenesis (reviewed in 2.1.3.0.2.). Alternatively, increased membrane phospholipid levels of EPA results in the synthesis of less pro-inflammatory mediators, eicosanoids of 3- and 5-series (Dommels et al., 2002).

ω-6 and ω-3 fatty acids are polyunsaturated fatty acids (i.e. contain two or more double bonds) named after the position of the first double bond in the carbon atom chain starting from the methyl end of the molecule. Unlike plants, most animal species, including humans, lack the enzymes required for linoleic (18:2 ω-6) and α-linolenic (18:3 ω-3) acid synthesis, so these PUFAs are essential, i.e. cannot be synthesized by the body, and therefore must be obtained from the diet (Hunt and Groff, 1990). Once consumed in the diet linoleic acid is converted by the body through various elongation and desaturation reactions into arachidonic acid (AA, 20:4 ω-6), while α-linolenic acid serves as the precursor of eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) (Figure 3.1.). It has been shown that activity of Δ6 desaturase, the main enzyme involved in desturation, has greater affinity for α- linolenic compared to linoleic acid and so increased dietary intake of α-linolenic acid can effectively decrease the formation of AA (Hunt and Groff, 1990). Moreover, AA and EPA also compete for the

Figure 3.1. Metabolism and nomenclature of the main polyunsaturated fatty acids of linoleic (ω-6) series and the α-linolenic (ω-3) series.

(Modifed from Roynette et al., 2004)

ω-6 and ω-3 fatty acids obtained from diet compete for metabolism by a series of common enzymes (desaturases and elongases). Twenty-carbon fatty acids of either family (ω-6 AA or ω-3 EPA) can act as precursors for different series of eicosanoids (including prostaglandins (PG), thromboxanes (Tx) and leukotreines (LT)) which are involved in modulation of inflammation and carcinogenesis. ω-6 AA derived eicosanoids are potent pro-inflammatory and pro-tumor mediators, while ω-3 EPA acts as a precursor of less potent eicosanoids.


ω-6 series
Linoleic acid
(18:2 ω-6)
Gamma-linolenic acid
(18:3 ω-6)
Dihomo-gamma-Linolenic acid
(20:3 ω-6)
COX
1-series
PG
Arachidonic acid (AA)
(20:4 ω-6)
LOX
COXs
2-series PG
2-series Tx
4-series LT
ω-3 series
α-Linolenic acid
(18:3 ω-3)
Δ 6 deasturase
(18:4 ω-3)
elongase
(20:4 ω-3)
Δ 5 deasturase
Eicosapentaenoic acid (EPA)
(20:5 ω-3)
LOX
COXs
3-series PG
3-series Tx
5-series LT
elongase
Docosapentaenoic acid
(22:5 ω-3)
Δ 6 deasturase
Docosahexaenoic acid
(22:6 ω-3)

cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, and since ω-3 FAs are preferentially used, increased intake of EPA inhibits synthesis of AA-derived pro-inflammatory eicosanoids.

The competitive relationship between ω-6 and ω-3 FAs suggests an important therapeutic potential of ω-3 FA in inflammatory disorders and cancer prevention. As previously reviewed (2.1.3.0.2.) suppression of AA derived eicosanoids with NSAIDs and COX-2 inhibitors showed a decrease frequency and number of premalignant and malignant lesions in majority of patients. However, not all patients equally respond or are equally suitable for a treatment with these drugs. In addition, the side effects associated with intake of these drugs eventually limit their usefulness. For these reasons, there is opinion that dietary intervention with ω-3 FAs, which is thought as more physiological and less invasive, may have the advantage over pharmacotherapy and serve more effectively in long-term preventive strategy.

3.1.0. Role of ω-3 FA in cancer prevention

Epidemiological studies have shown that cancer rates in Greenland Eskimos, who consume a large quantity of seafood rich in EPA and DHA, are relatively low considering their total fat intake. This is in contrast with the predominance of ω-6 FAs in a Western diet, which has been associated with increased risk for colon cancer (Bang et al, 1980). Studies on experimentally induced carcinogenesis demonstrated the tumor-promoting role of ω-6 PUFAs and protecting role of ω-3 PUFAs (Akihisa et al., 2004; Reddy and Sugie, 1988; Reddy and Maruyama, 1986). Supplementation with either perilla oil (rich in α-linolenic acid), DHA, or fish oil demonstrated decrease in total number and

multiplicity of ACF (Dommels, 2002; Good et al., 1998). Examination of data from 24 European countries indicated that a low ratio of ω-3 to ω-6 FAs in the diet is a risk factor for colon cancer (Caygill and Hill, 1995). Anti et al. (1994), showed that after six months of fish oil supplementation there was a significant increase in EPA and decrease in linoleic and AA in colonic mucosal lipids. Fish oil supplementation 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). Case-control studies have found an inverse, but not significant, correlation between fish oil and colon cancer incidence (Caygill et al., 1996; Hursting et al., 1990; Willet et al., 1990). The role of PUFAs in modulation of colitis-associated carcinogenesis, however, has never been investigated.

3.1.1. Role of ω-3 FA in prevention of ulcerative colitis

Some epidemiological studies reported that increased consumption of fat, including *trans* fatty acids (chemically processed hydrogenated fats), saturated or unsaturated fat, may be important in etiology of UC, while others reported a lack of association between intake either in the amount or type of these fats and UC incidence (reviewed in Cashman and Shanahan, 2003). Recently, there is emerging evidence that nutritional intervention with ω-3 FAs is effective in the treatment of IBD. These observations correlate with the evidence that metabolites of AA are key mediators of inflammation in IBD (De Dombal et al., 1993). A Japanese population-based study demonstrated a positive correlation between increased intake of ω-6 FAs in relation to ω-3 FAs and increased incidence of CD (Shoda et al., 1996). Most of the clinical studies

concluded that addition of high levels of fish oil to the diet of UC patients exert a range of beneficial effects as seen in suppression of immune reactivity, reduction in disease activity and symptomatology, steroid-sparing effect, and a delay of the first episode of relapse (reviewed in Head and Jurenka, 2003; Belluzzi et al., 2000; Endres et al., 1999). In rats pre-treated with ω-6 or ω-3 PUFAs the induction of colitis caused significantly less colonic damage in a group supplemented with ω-3 PUFAs, as compared to the ω-6 supplemented group (Salas et al., 1990). Nieto and colleagues (2002), showed that rats with experimentally induced UC had significantly less macroscopic and microscopic colonic damage as well as lower PGE2 levels after 1 week of feeding high fish oil diet compared to rats fed either high fat olive oil (monounsaturated FA) or pig brain phospholipids diet (source of ω-6 and ω-3 PUFAs).

Suggested mechanisms by which ω-3 FAs may ameliorate intestinal inflammation include reduced production of potent AA-derived inflammatory mediators (PGE2, TXA2, LTB4) and inhibition of cytokines with pro-inflammatory cellular action such as TNF-α and IL-1β (Calder, 1998). Moreover, supplementation with ω-3 FAs has been shown to reduce several aspects of neutrophil, monocyte and lymphocite functions (Kelley, 2001; Calder, 1998). These observations indicate that dietary intervention with ω-3 FAs might be promising in modulating the immune and inflammatory responses in patients with UC.

3.1.2. Dietary sources of ω-6 and ω-3 fatty acids

Since ω-6 and ω-3 PUFAs are essential FAs they must be obtained through food sources. Plants seeds and leaves are the richest sources of ω-6 PUFAs. Linoleic acid is the main constituent in most vegetable oils, including corn, safflower, soybean and

sunflower (Dommels et al., 2002). The ω-3 PUFAs are found in phytoplankton in long chain forms (EPA and DHA) and in small amounts in chloroplast of green plants in a form of α-linolenic acid. Through the food chain, all forms of marine life get enriched with long chain ω-3 PUFAs (Woutersen, et al., 1999). The best sources of α-linolenic acid are flaxseed (linseed) oil, perilla oil, and walnuts. In smaller amounts α-linolenic acid is also present in dark green leafy vegetables (Dommels et al., 2002).

Most of the studies that investigated effects of ω -3 FAs on inflammatory process or carcinogenesis were carried out either with fish oil or with isolated long chain PUFAs, EPA or DHA. The therapeutic potential of α-linolenic acid, which is a precursor of EPA and DHA, might itself be very beneficial, however, has been much less studied. It is speculated that α-linolenic acid should exert similar effects as EPA and DHA. If that proves correct, the usage of ω -3 FAs in dietary manipulation and pharmaceutical application would be much easier, since a higher saturation level of α-linolenic acid makes it nine times more resistant to the oxidation process as compared to EPA and DHA (Narisawa et al., 1994).

3.2. Preliminary study

There have been no studies investigating the modulating effect of dietary lipids on development of colon cancer under inflammatory conditions. The preliminary study performed in our laboratory to assess the effects of low and high levels of ω-6 and high level of ω-3 PUFAs on development of preneoplastic colonic lesions in the presence and absence of inflammation was based on the hypothesis that dietary intervention with a high flaxseed oil diet, due to its high content of α-linolenic acid (18:3 ω-3) will reduce

inflammation, which, in turn, lowers the heightened risk of developing colon cancer, whereas a high corn oil diet rich in linoleic acid (18:2 ω-6) will not (Dahleh, 2001). The reasoning behind this was that an abundance of α-linolenic acid (18:3 ω-3), since it is preferentially used by desturase enzymes, would suppress production of potent pro-inflammatory and tumor promoting eicosanoids derived from AA (20:4 ω-6).

During the preliminary experiment, male Sprague-Dawley rats were fed three diets, two high fat diets varying in the level of ω-3 or ω-6 fatty acids along with a group fed a low fat diet (5% corn oil). Rats were fed diets with (LFC+, HFF+, HFC+) or without (LFC, HFC, HFF) carrageenan (2% by weight) for two weeks prior to injection with azoxymethane (15mg/kg) and for four weeks after the injection. After CO_2 asphyxiation their colons were evaluated for aberrant crypt foci (ACF). The results of this study confirmed previous findings, which showed that in the absence of inflammation high fat ω-6 diet promotes ACF development while high ω-3 as well as low ω-6 diets have protective effects. In the presence of inflammation, however, both high fat diets (HFC+ ω-6 and HFF+ ω-3) reduced the growth and number of ACF, while low fat LFC+ ω-6 diet promoted it (Figure 3.2.). The most noteworthy observation was that in inflamed colon high fat ω-6 diet, which normally (in the absence of inflammation) promotes the growth of ACF or tumors, acted in an opposite manner and similar to a high fat ω-3 diet in reducing the number of ACF (Table 3.2.) (Dahleh, 2001). These findings support the potential of dietary lipids to serve as preventive agents in reducing the associated risk of developing colon cancer in UC patients. However, these findings also raised an important question: is it a high amount of fat in the diet or the type of fat (PUFAs) that has

Table 3.2. Total and number of ACF by crypt multiplicity in male Sprague-Dawley rats fed one of three diets: LFC, HFC or HFF with or without carrageenan 2 weeks prior and 4 weeks after AOM injection.

Diet group	TACF	ACF1	ACF2	ACF3
LFC	147.9 ± 21.5	112.2 ± 13.4	31.1 ± 6.9	4.4 ± 2.6
LFC+	158.7 ± 22.7	98.2 ± 14.2	44.5 ± 9.2	10.0 ± 4.1
HFC	177.0 ± 23.7	119.7 ± 13.9	48.7 ± 11.8	7.1 ± 2.3
HFC+	103.9 ± 17.0	74.4 ± 12.9	25.5 ± 5.6	3.2 ± 1.2
HFF	147.1 ± 24.4	102.6 ± 17.1	39.1 ± 8.3	5.4 ± 1.8
HFF+	100.0 ± 16.9	73.8 ± 11.7	21.6 ± 5.6	2.8 ± 0.8

Values are means ± SE. There was no significant difference among groups at $P \leq 0.05$ (ANOVA and Tukey's test). (Data obtained from Dahleh, 2001).

alleviating effect on colitis-associated carcinogenesis?

3.3. Summary

Human as well as animal studies have demonstrated over-expression of AA-derived eicosanoids (PGE2, PGI2 and TxA2) in UC as well as in colonic adenomas and carcinomas. There is increasing evidence that these key mediators of inflammation also exert various effects favourable to cancer development including mutagenic ability, stimulation of cell proliferation, induction of cytokines synthesis, induction of angiogenesis, immunosuppression, promotion of metastasis, and inhibition of apoptosis.

The polyunsaturated fatty acid (PUFA) composition of cell membranes is to a great extent dependent on the dietary intake. Because it normally predominates in the diet and thus in cell membrane, AA is the principal precursor for eicosanoid synthesis. However, by increasing dietary intake of ω-3 FAs, membrane levels of AA decrease while levels of EPA and DHA increase resulting in synthesis of less pro-inflammatory mediators. Numerous evidences indicate that dietary lipids modulate many critical signalling enzymes in the carcinogenic process. Experimental studies investigating the modulating role of high fat diet in colon carcinogenesis demonstrated the tumor-promoting role of saturated and ω-6 PUFAs and protecting role of ω-3 PUFAs. Furthermore, with few exceptions, supplementation with fish oil in UC patients has shown promising therapeutic potential of dietary intervention with ω-3 FAs.

To our knowledge, the role of dietary lipids in modulating the effect of inflammation and its associated risk of developing colon cancer has not been investigated. The preliminary data from the study in our laboratory showed beneficial effects of both, ω-6 and ω-3, high

fat diets in reduction of the number and growth features of ACF in colitis-associated carcinogenesis model. The low fat ω-6 diet, however, showed as tumor promoting. It is not clear if these unexpected findings are the effect of high fat diet per se or the type of fat used. If confirmed, these findings could be applied as important means in prevention of colon cancer development in UC patients.

CHAPTER 4

MATERIALS AND METHODS

In this chapter materials and methods used in the study are described. Unless otherwise specified all chemicals were purchased from Sigma Chemical Co., Mississauga, Ontario, Canada.

Animals

Female, Sprague-Dawley rats (purchased from Charles River, Montreal, Canada) weighing approximately 75-80g, were acclimatized for 1 week on standard rat chow before initiation of the experiments. Animals were housed, two to three per cage, in wire meshed stainless steel cages with sawdust bedding in a room under controlled environmental conditions (21°C and 50% humidity) and photoperiod (12 hr light/dark cycle). All procedures involving the animals were approved by the University of Manitoba Animal Care Committee and strictly adhered to the guidelines of the Canadian Council of Animal Care. During the entire experimental periods animals had access to food and water *ad libitum*.

Diet

Cornstarch was purchased from the University of Manitoba Food Service. Flaxseed oil was purchased from Omega Nutrition (Vancouver, BC) and corn oil from Superstore (Mazola, Etioboke, ONT). All other dietary ingredients were purchased from Harlan Teklad (Madison, WI).

All diets used in this study were based on a semi-purified AIN 93G (American Institute of Nutrition, 1993) standard diet with modifications specified in Appendix A. Four types of high fat diets varying in fatty acid composition were employed. High fat corn oil (HFC) diet contained 23.8% of corn oil by weight. High fat olive oil (HFO) and high fat beef tallow (HFB) diets contained 5% of corn oil and 18.8% of respective fat by weight. The high fat flaxseed oil (HFF) diet was, however, 14.3% corn oil and 9.5% flaxseed oil by weight. Flaxseed oil is composed roughly of 73% of unsaturated fatty acids; therefore, this oil is at a higher risk of rancidity compared to other lipids used. For this reason, the HFF diet's 23.8% fat composition was split into 2:3 (flaxseed oil:corn oil) ratio. The low fat corn oil (LFC) diet, used as a control in all experiments, contained 5% corn oil by weight. Furthermore, the amount of cellulose, vitamin mix, mineral mix and casein in the diets was adjusted to ensure that animals were fed isocaloric diets. Fresh diets were prepared weekly and stored at 4°C in dark. Oils were stored in amber colored bottles at 4°C to avoid rancidity and oxidation.

Carrageenan (CAR)

The sulphated polysaccharide, commercial grade Type-I (predominantly kappa) carrageenan (Sigma Chemical Co., St. Louis, MO, USA), was used to induce colitis in the rats. Carrageenan was added undegraded 2% by weight to the experimental diets.

Azoxymethane (AOM)

AOM, a colon specific carcinogen, was diluted in 0.9% saline and injected s.c. in the rats at a concentration of 10 mg/kg body weight.

Body weight

All animals were regularly weighed, starting at the beginning of the experiment, once per week throughout the experiment, and at the termination.

Complete blood count (CBC)

Animals were fasted overnight (at least 12 hours) prior to termination, but had free access to water. After termination by CO_2 asphyxiation, blood was drained by cardiac puncture, stored at 4°C in blood tubes with liquid EDTA (K3) (Fisher Scientific, Ottawa, ON, Canada), and sent to the Regional Health Sciences Centre, Manitoba, Canada for complete blood count analysis. CBC was performed using standardized procedure and employing rat specific software.

Tissue preparation

After termination by CO_2 asphyxiation, the colons were removed, flushed with ice cold PBS, placed on a cold plate (4°C), and slit longitudinally from the ceacum to the anus. Colons intended for ACF scoring were fixed flat between filter papers in 70% ethanol, coded for blind scoring and stored at 4°C. Colons intended for other histological examinations were cut open rolled from rectal to ceacal end into so called "swiss rolls", fixed in 10% neutral buffered formalin and stored at 4°C. Mucosal scrapings were collected from those colons intended for biochemical analysis. Scrapings were placed into sterile cryo-vials, frozen immediately in liquid nitrogen and stored at –80°C.

1. Histological analysis

a) Quantification of ACF

After a minimum of 24 hours fixation in 70% ethanol, unsectioned colons were stained for 5-10 minutes in 0.2% methylene blue dissolved in PBS (Appendix B). The colons then were placed on microscopic slides and viewed (scored) mucosal side up, in 2 cm sections starting from the rectal to the ceacal end, using a light microscope at magnification of x100. ACF were distinguished from normal crypts by their increased size, elongated luminal opening, increased thickness of the epithelial lining and increased pericryptal zone as described previously (1.2.2.2.). The number and multiplicity of ACF were determined along the entire length of the colon. Average crypt multiplicity was determined as the mean number of crypt/focus/colon. ACF with different growth features were grouped based on their crypt multiplicity into small (1-2 crypts per focus) and large (advanced) ACF (more than 3 crypts per focus).

b) Immunohistochemistry

Excised colons, washed with ice cold 1X PBS, longitudinally cut open and rolled into swiss roles, were fixed in 10% neutral buffered formalin. The fixed swiss rolls of colons were embedded in paraffin wax, sectioned longitudinally at a thickness of 4-6 μm, and mounted onto slides. One section from each group was stained with hematoxylin and eosin (H&E), while others were processed for immunoperoxidase staining. Immunoperoxidase staining was carried out using the mouse Histostain-Plus Peroxidase

bulk kit from Zymed (San Francisco, CA, USA) or goat ABC Staining System (Santa Cruz Biotechnology, CA, USA).

Tissue sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, incubated for 10 minutes in 3% H_2O_2 to quench endogenous peroxide. Sections then were washed in 1X PBS and incubated with normal blocking serum for 20 minutes to block non-specific binding. One of the three primary antibodies used: mouse monoclonal NOS-2 (sc-7271), goat polyclonal COX-2 (sc-1745) or goat polyclonal TNF-α (sc-1350) (all purchased from Santa Cruz Biotechnology, CA, USA), was diluted in PBS (1:40) applied to the sections and incubated for 2 hours in a humid chamber at room temperature. The sections then were incubated with respective biotinylated secondary antibody (linking reagent) for 20 minutes, followed by incubation with avidin biotin enzyme reagent (labelling agent) for 20 minutes. The peroxidase reaction was initiated by immersing the slides in 0.06% 3,3-diaminobenzidine tetrahydrochloride substrate (Zymed, San Francisco, CA, USA) for 3-10 minutes i.e. until desired stain intensity developed. The slides with sections were rinsed 3 times with fresh water and counter stained with giemsa (Sigma Chemicals, St Louis, MA, USA). Sections were then sequentially dehydrated and mounted with Permount (Fisher Scientific, Ottawa, ON, Canada). Incubations throughout the procedure were carried out in a humid chamber at room temperature, and slides with sections were washed extensively with fresh 1X PBS between incubations. To confirm the specificity of the antibodies, two slides with colonic sections were simultaneously processed in a similar manner as mentioned earlier for the test slides, except for the omission of either primary or secondary antibodies, respectively. All slides were coded and then evaluated for protein.

2. Biochemical Analysis

a) Protein Analysis

For protein content determination, colon scrapings were removed from – 80°C. Scrapings were homogenized in 2 ml of ice-cold homogenizing buffer (pH 7.5) (Appendix C) for 30 seconds using a polytron homogenizer in an ice-bath. PMSF protease inhibitors (1 μl) was added to homogenate and ultracentrifuged for 20 minutes at 10, 000 RPM. Aliquots of the supernatant drawn were taken for protein analysis and the remaining was labelled and immediately frozen at –80°C. The total protein was quantified according to the method of Bradford using a Coomassie protein assay reagent (Pierce, Rockford, IL, USA), and bovine serum albumin as the standard. Samples were analyzed in duplicate using a Spectra Max 3000 (Molecular Devices, Sunnyvale, CA, USA).

Western Blot

Whole homogenates of colonic mucosae were prepared and their protein concentration assessed. To avoid overloading of the gel and to ensure the best band readings the amount of protein loaded for each antibody was determined based on the median of the linear range (increasing total protein concentrations of the homogenates were run to establish a linear range). A positive control was included in all the gels to minimize effects of the background and gel-to-gel variability. Volumes of colonic mucosal homogenates containing 50 μg of protein (for TNF-α and iNOS) and 40 μg of protein (for COX-2) were heated for 4 minutes at 80°C with 2X SDS sample buffer, containing 5% (vol/vol) β-mercaptoethanol, 10% (wt/vol) glycerin, 0.001% (wt/vol)

bromophenol blue and 63 mM/L Tris-HCl (pH 6.8); and were separated by 10% SDS-PAGE for 70 minutes at 170 volts using the Mini-Protean Bio-Rad-II system (Bio-Rad Laboratories Ltd, Canada). The separated proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Life Technologies, IL, USA) in a 20% methanol buffer for 120 minutes at 120 volts and at a temperature of 4°C. Membranes were blocked for 1 hour at room temperature with 5% skim milk powder in TBS-T (tris buffered saline with 0.1% Tween-20), and then probed with primary antibodies at 4°C overnight on a shaker. Primary antibodies (purchased from Santa Cruz Biotechnologies, CA, USA) were goat anti-COX-2 (sc-1745) and goat anti-TNF-α (sc-1350) at dilutions 1:300 and 1:100 respectively; and mouse anti-iNOS (sc-7271) antibody at 1:200 dilution. After incubation with primary antibodies blots were washed with TBS-T for 40 minutes and incubated with appropriate secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. Secondary antibodies used were anti-goat IgG peroxidase conjugate (product # A 9452, Sigma Chemical Co., Mississauga, ON, Canada) at dilutions 1:5000 and 1:10,000 for COX-2 and TNF-α respectively, and anti-mouse IgG, HPR- linked antibody (# 7076, Cell Signaling Technology Beverly, MA, USA) at dilution 1:1000 for iNOS. After washing for 40 minutes in TBS-T, the blots were incubated with ChemiGlow™ Chemiluminiscent Substrate (Alpha Innotech Corporation, CA, USA) for 5 minutes and exposed in a FluorChem™ Imaging Systems (Alpha Innotech Corporation, CA, USA). Densitometric analysis was conducted on protein bands visible at particular molecular weights (19kDa, 130kDa and 74kDa, to detect TNF-α, iNOS and COX-2 respectively) using the FluorChem™ Imaging Systems (Alpha Innotech Corporation, CA, USA). In order to monitor equal loads and even

transfer of proteins, the gels were stained with Coomassie blue, de-stained and checked. The buffer systems and gel recipes used in the Western blot (Appendix D) were based on the method of Laemmli.

b) Analysis of dietary lipids

GC (Gas Chromatography)

Fatty acids composition of dietary lipids used in experimental diets was analysed using gas chromatography (GC) according to the method described in AOCS (American Oil Chemists' Society, volume I, 1998 – Official methods and recommended protocols, pp CE 1-62). In short: Samples of all diets used in the study (LFC, HFC, HFO, HFB, HFF) plus three random repeats (LFC, HFF and HFC) used as a control, underwent fat extraction through three extraction/evaporation cycles. The extracting solvent consisted of HPLC grade chloroform and methanol (1:1, v/v) (Fisher Scientific Limited, Nepean, ON, Canada). After evaporation, the extracted fat was weighted and the total amount of fat per sample calculated. Iso-octane (1 µl), which contained exact amounts of internal standard (C17:1), plus 12 ml of 2% H_2SO_4 in methanol was added into 50 µl of each sample, vortexed and heated (2 hr at 65-70°C) until a monophase system was achieved. After cooling to room temperature, 6 ml of iso-octane and 6 ml of distilled H_2O was added, mixed well, and 1 ml of clear upper layer transferred into chromatographic GC-vials (first vial contained pure standard). Esterified samples were automatically run on GS overnight, and chromatograms assessed for presence of individual FAs as based on calculation with internal standard.

Statistical analysis

Statistical analysis of the data was carried out using Analysis of Variance (ANOVA) in conjunction with Tukey's Test using SAS statistical software package for microcomputers (SAS Institute Inc., Cary, NC). P value ≤ 0.05 was considered significant for all test.

CHAPTER 5

Modulation of precancerous lesions and protein expression of TNF-α, iNOS and COX-2 in colonic mucosae by inflammation caused by carrageenan feeding for different durations

5.0. Introduction

Colon cancer is the third most frequently diagnosed cancer and the second leading cause of cancer mortality among man and women in the developed world (Franks and Teich, 2001; Smalley and DuBois, 1997). Ulcerative colitis (UC) is a chronic inflammatory bowel disease of unknown etiology characterized by diffuse mucosal inflammation limited to any part or the entire colon. For yet unexplained reasons, ulcerative colitis is more common in people who live in northern climates, in developed countries, and in urban areas. In Manitoba, the overall annual incidence rate reported for year 2000 was 15/100,000 and the prevalence rate was 234/100,000, which are among the highest reported in the world (Bernstein, 2004).

A substantial body of evidence supports the hypothesis that chronic inflammation may play a causative role in a variety of cancers. The association between UC and colorectal cancer was first reported at the beginning of the 20th century (Tsianos, 2000). Ulcerative colitis is associated with an increased risk of the development of colonic epithelial cells-derived neoplasia. One of the most often reported risks for cancer development in UC patients is the long duration of the disease. UC-associated colon cancer is only rarely encountered when total duration of disease is less than 8-10 years, but thereafter the risk rises at approximately 0.5-1% annually (Eaden and Mayberry, 2000). Although it is likely that colorectal cancer development is related to the length of

the underlying inflammatory process, to our knowledge, no study has convincingly shown that duration of inflammation plays a critical role in cancer development.

The involvement of pro-inflammatory molecules TNF-α, iNOS and COX-2, in regulation of several processes that are shown to enhance inflammation as well as initiate and promote tumor growth, has been confirmed by many experiments. However, the exact mechanism and understanding of the timing of individual molecular events that predispose neoplastic transformation in colitis affected colons remains obscure.

An ongoing interest in our laboratory has been to identify an experimental approach that would induce mild chronic colitis-like changes that closely resembles human disease (at any point in time 80% of UC patients are either asymptomatic or have mild symptoms (see 2.0.2)), allow additional application of carcinogen with low level of discomfort and subsequent long term animal survival. Our preliminary research showed that feeding undegraded carrageenan (2% by weight) caused a number of mild chronic-colitis like changes; however, it remained unclear as to when visible morphological changes occur and if morphological changes precede biochemical changes.

The main objectives of this study were:

1. To sequentially analyze the inflammatory effect of carrageenan in rat colonic mucosae in order to determine the duration required to produce morphological and biochemical changes (Experiment 1).
2. To assess the effect of the duration of CAR feeding before (Experiment 2A) and concurrent (Experiment 2B) with the administration of carcinogen injection on the sensitivity of colonic mucosa to AOM induced colon carcinogenesis.

5.1. Materials and methods

Female, Sprague-Dawley rats (Charles River, Montreal, Canada) weighing approximately 75-80g, were acclimatized for 1 week on standard rat chow. Animals were housed, two to three per cage, in wire meshed stainless steel cages with sawdust bedding in a room under controlled environmental conditions (21 °C and 50% humidity) and photoperiod (12 hr light/dark cycle). All procedures involving the animals were approved by the University of Manitoba Animal Care Committee and conform to the guidelines of the Canadian Council of Animal Care. Diet was based on modified AIN-93 diet containing 5% corn oil. Undegraded Commercial Type I Carrageenan (Sigma Chemical Co.) was added to the diet at 2% by weight. Animals had access to food and water *ad libitum*. Initial, weekly, and body weights of animals at termination were recorded. The consistence (loos stool or diarrhea) and content of stool (macroscopic presence of blood or mucus) were checked daily.

Detailed description of materials and techniques used in this study is provided in Chapter 4.

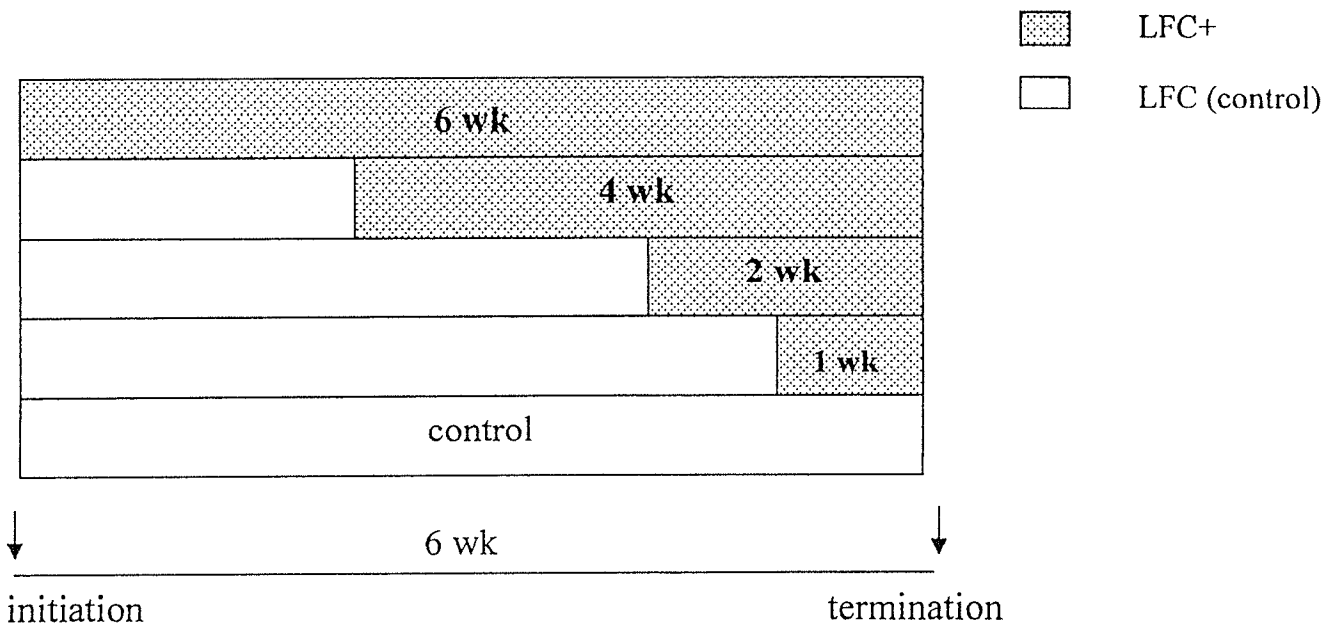
5.1.0. Study design

After a period of acclimatization rats were randomly allocated into experimental groups. Two experiments were conducted:

Experiment # 1:

See Figure 5.1. for the schematic representation of the experimental design. To assess the effect of feeding CAR for 0 (control), 1, 2, 4 or 6 weeks on colonic morphology and generation of molecules associated with inflammatory process, rats were

Figure 5.1. Schematic representation of the experiment 1



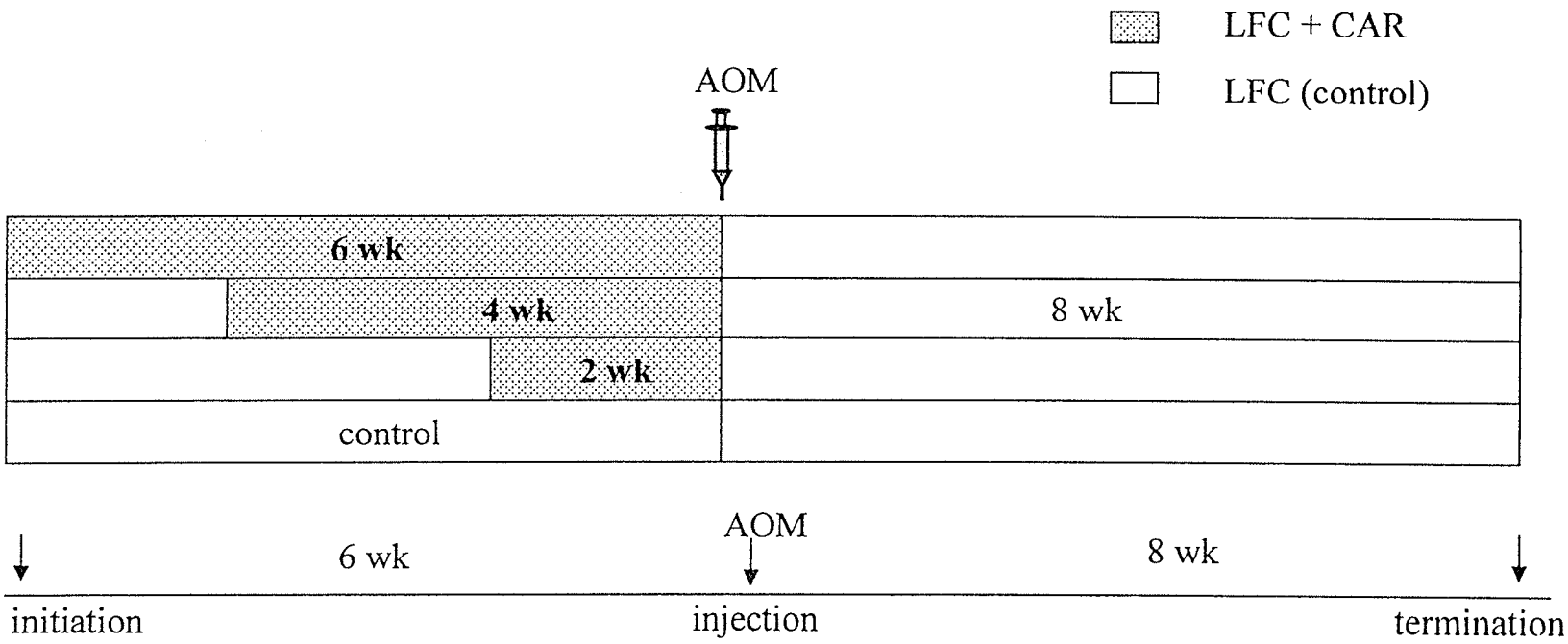
Low fat corn oil diet = LFC
LFC + carrageenan = LFC+

randomly allocated into 5 groups. "Control group" received a low fat corn oil diet (LFC) for 6 weeks. "1 wk group" received LFC diet for 5 wk and than switched to a low fat corn oil diet with carrageenan (LFC+) for 1 wk. "2 wk" and "4 wk" groups received LFC diet for 4 and 2 weeks and than switched to LFC+ diet for 2 and 4 weeks respectively. "6 wk group" received LFC+ diet for the whole duration (6 weeks). At the end of the study all animals belonging to the five groups were at the same age. Animals were terminated with CO_2 asphyxiation. Blood obtained by cardiac puncture *post mortem* (4 animals/group) was sent for the complete blood cell count (CBC) analysis. Colons of 3 animals from each group were excised, rolled up into, so called, "swiss rolls" and used for immunohistochemistry. Colonic mucosa (6 animals/group) from 2 wk, 6 wk and control group, were processed to assess the levels of tumor necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).

Experiment # 2:

A) See Figure 5.2. for the schematic representation of the experimental design. To determine if duration of inflammation before introduction of carcinogen would alleviate the risk of developing colon cancer in the carrageenan induced animal model of inflammatory bowel disease 32 animals (8/group) were fed a low fat diet (5% corn oil) with carrageenan (LFC+) or without carrageenan (LFC) as follows: "2 wk group" received LFC diet for 4 weeks and switched to LFC+ diet for next 2 weeks. The "4 wk group" received LFC diet for 2 weeks and then was switched to LFC+ diet for additional 4 weeks. "6 wk group" and "control group" received LFC+ or LFC diet respectively for the entire duration of the experiment (6 weeks). At the end of 6 wk all animals were injected s.c with AOM (10mg/kg) and switched to LFC diet for 8 wk. All rats were

Figure 5.2. Schematic representation of the experiment 2A

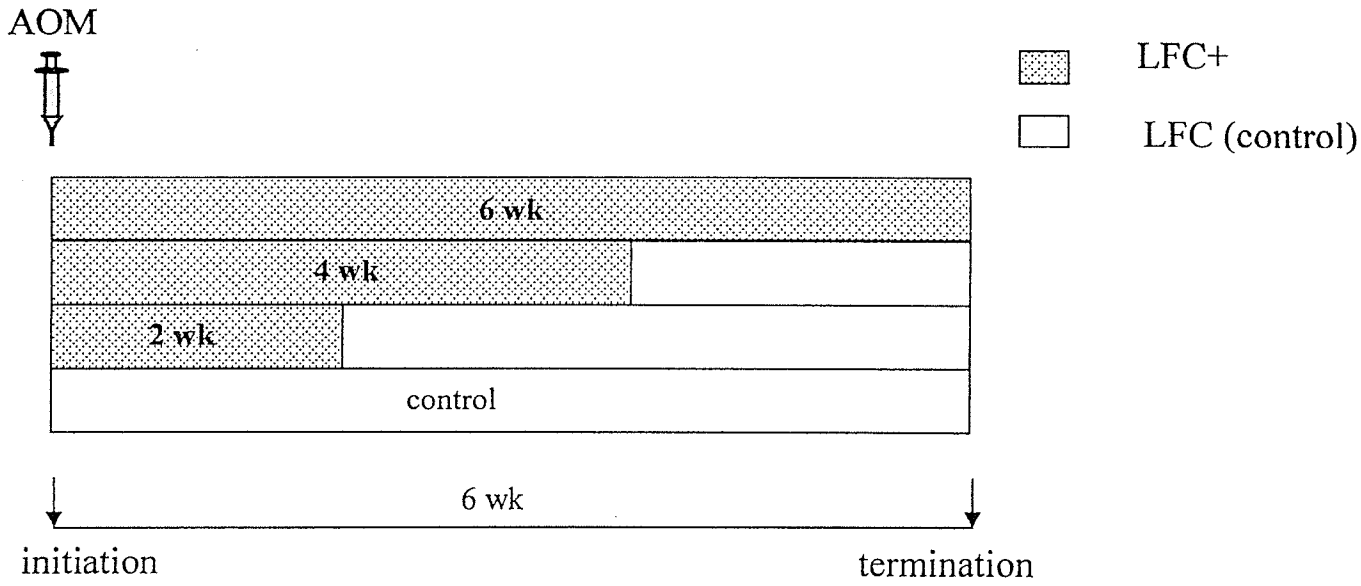


Low fat corn oil diet = LFC
LFC + carrageenan = LFC+
Azoxymethane = AOM

terminated with CO_2 asphyxiation and their colons were evaluated for aberrant crypt foci (ACF).

B) See Figure 5-3, for the schematic representation of the experimental design. The objective was to determine if initiation of inflammation by feeding CAR concurrent with carcinogen injection, and the duration of inflammation after injection, affects the risk of developing colon cancer. Thirty-two rats (8 animals/group) were randomly allocated into four groups and injected with AOM (10mg/kg). At the same time low fat corn oil diets with (LFC+) or without (LFC) carrageenan, were introduced as follows: "2 wk group" received LFC+ diet for 2 weeks and then was switched to LFC diet for 4 weeks. "4 wk group" received LFC+ diet for 4 weeks and then was switched to LFC diet for 2 weeks. "6 wk group" and "control group" received LFC+ or LFC diet respectively for the entire duration of the experiment (6 weeks). After the end of six weeks all animals were terminated with CO_2 asphyxiation and their colons used for ACF analysis (8 animals/group).

Figure 5.3. Schematic representation of the experiment 2B



Low fat corn oil diet = LFC
LFC + carrageenan = LFC+
Azoxymethane = AOM

5.2. Results

Experiment 1:

During the experiment changes in stool consistency or macroscopic presence of blood or mucus were not observed. There were no significant differences in the mean weights of animals treated with CAR for different periods of time compared to the controls (Table 5.1.).

Haematological assessment showed that after 1 wk of CAR feeding there was an increase in platelets (PLTs) and a decline in mature neutrophils compared to the control. With longer feeding, the count of these cells started to change and in the 6 week group both cell types reached their normal levels. The lymphocyte number decreased in CAR treated animals and then it increased in 6 wk group, while the number of monocytes seemed to increase with increasing duration of CAR feeding reaching 2-fold higher levels in the 6 week group compared to the control group (Table 5.2.).

Within two weeks of initiation of the LFC+ diet, the number of lymphoid nodes and patches in the colonic mucosa of stained sectioned "swiss-rolled" colons increased (Figure 5.4.). Morphological features specific for colonic mucosae during the inflammatory process, such as migration of lymphocytes into lamina propria, dilated capillary network, patchy destruction with loss of surface epithelium and crypts, fibrotic changes and lymphocytes infiltration in the lamina propria, were observed in colonic tissues of 4 and 6 weeks groups as depicted in Figure (5.5.).

Table 5.1. Average body weights (g) of female Sprague-Dawley rats fed low fat corn oil diet with or without carrageenan for different durations.

Experimental groups	Animal weights in grams (Mean ± SE)
control	270.4 ± 12
1 wk	256.3 ± 7
2 wk	259.7 ± 18
4 wk	260.8 ± 9
6 wk	247.8 ± 12

1. Animals (n = 4/group) were fed LFC+ diet for the time periods of 1, 2, 4 and 6 weeks. Control animals were fed LFC diet for 6 weeks.
2. There was no significant difference among the weights of the animals at $P \leq 0.05$ (ANOVA and Tukey's test).

Table 5.2. The effect of carrageenan feeding on blood cell counts.

Cell count	Control group	1 wk group	2 wk group	4 wk group	6 wk group
PLT 10(9)/L	584 ± 134	711.33 ± 26	733 ± 51	669 ± 22	589 ± 99
Neut. Mature	0.7 ± 0.2	0.44 ± 0.3	0.6 ± 0.1	0.83 ± 0.3	0.71 ± 0.1
Lymphocyte	9.69 ± 1.3	8.12 ± 1.0	7.74 ± 0.3	7.31 ± 1.4	10.45 ± 1.7
Monocyte	0.22 ± 0.1	0.28 ± 0.0	0.14 ± 0.1	0.34 ± 0.2	0.48 ± 0.2

1. Blood was taken with cardiac puncture at termination.
2. Abbreviations are as follows: PLT (platelets); Neut. (Neutrophils).
3. Values (n = 4/group) are expressed as means ± SE; There were no significant differences among the same cell types across different treatment groups at $P \leq 0.05$ (ANOVA and Tukey's test).

Figure 5.4. Average number of lymphocytic infiltrations and Peyer's patches.

Values are means ± SE. Bars labeled with the same letter are not significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).

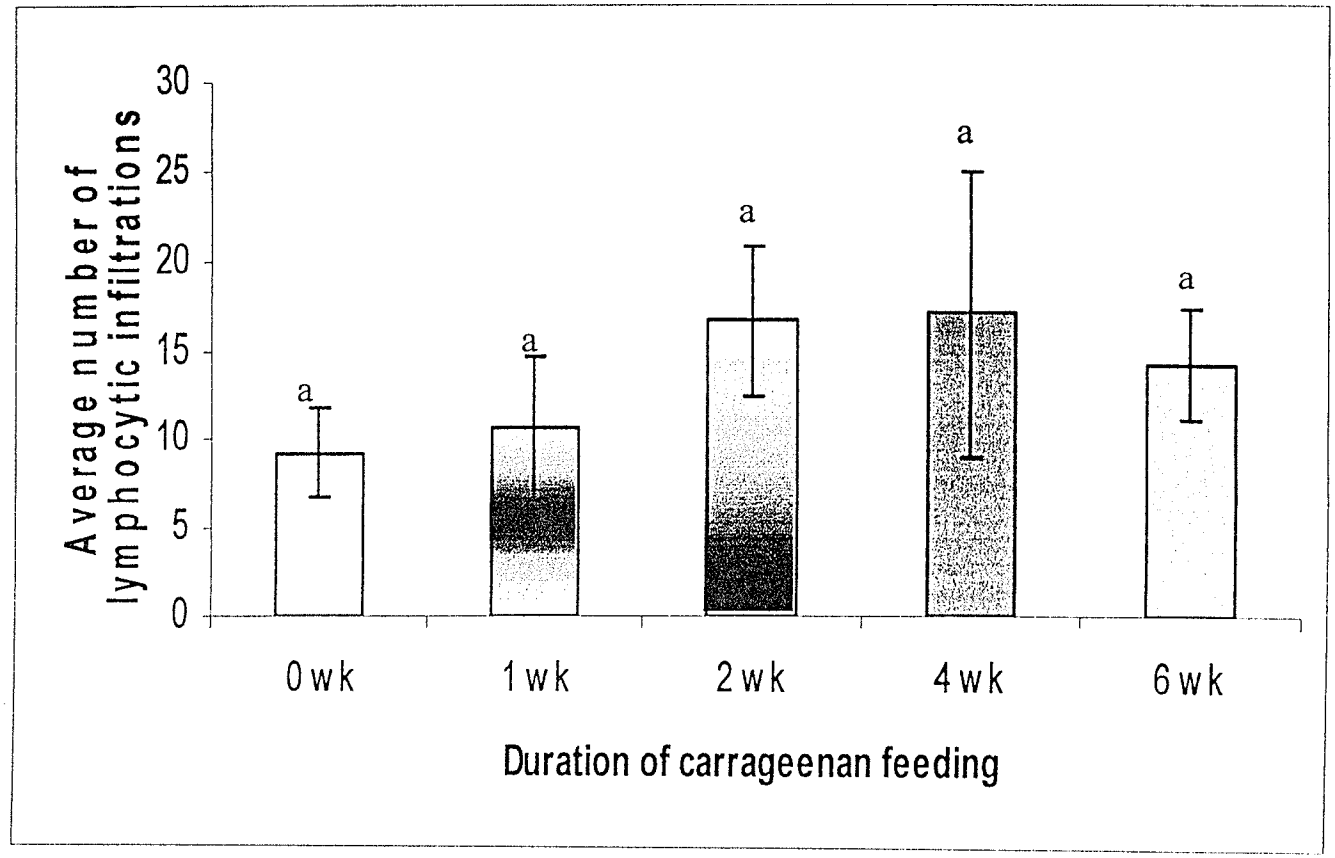
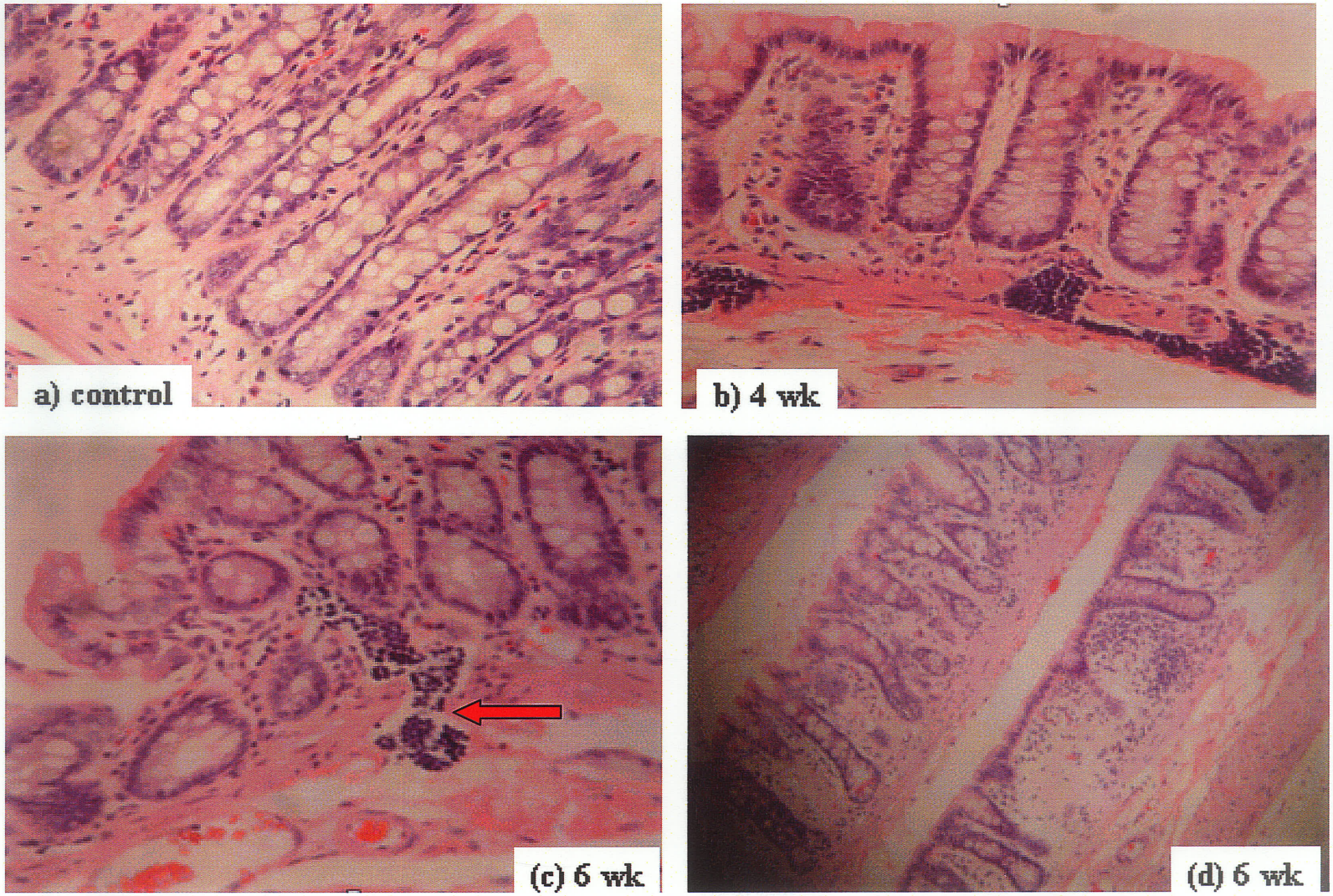

Average number of lymphocytic infiltrations
30
25
20
15
10
5
0
a
a
a
a
a
0 wk
1 wk
2 wk
4 wk
6 wk
Duration of carrageenan feeding

Figure 5.5. Morphological changes in colonic mucosa caused by carrageenan feeding. Longitudinal sections of colinic mucosae (H&E stained) without (a) and after carrageenan feeding for 4 weeks (b) and 6 weeks (c and d). Magnification: x400 (a, b and c) and x100 (d).

In non-carrageenan fed animals (a) the colonic mucosa had straight, long, parallel and evenly spaced crypts. In colonic mucosa after 4 weeks of carrageenan feeding (b) crypts appeared wider, shorter, with more cells per crypt. There was an increase in the size of the intercryptal zone also showing lymphocytic infiltration.

Note a cluster of lymphocytes (arrow) migrating into the lamina propria as well as dilated capillary network 6 wk after carrageenan feeding (c). The features of chronic colitis are noted, such as lymphocytic infiltration, fibrotic changes, irregular size crypts, and markedly increased intercryptal zones possibly due to focal necrosis (d).


a) control
b) 4 wk
(c) 6 wk
(d) 6 wk

Western blot analysis of colonic mucosa demonstrated that the COX-2 (72kDa) level declined with an increasing duration of CAR feeding and inflammation, so the highest expression was detected in the control group whereas the lowest expression was detected in the 6 wk group (Figure 5.6.). Two other enzymes, TNF-α (19kDa) (Figure 5.7.A and 5.7.B) and iNOS (130kDa) (Figure 5.8.A and 5.8.B), showed a significant decline at 2 wk after CAR feeding; with prolonged CAR feeding (6 wk), however, their levels increased compared to the control.

Figure 5.6. COX-2 expression in colonic mucosa as modulated by duration of carrageenan feeding. Values are means ± SE. Bars labeled with the same letter are not significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).

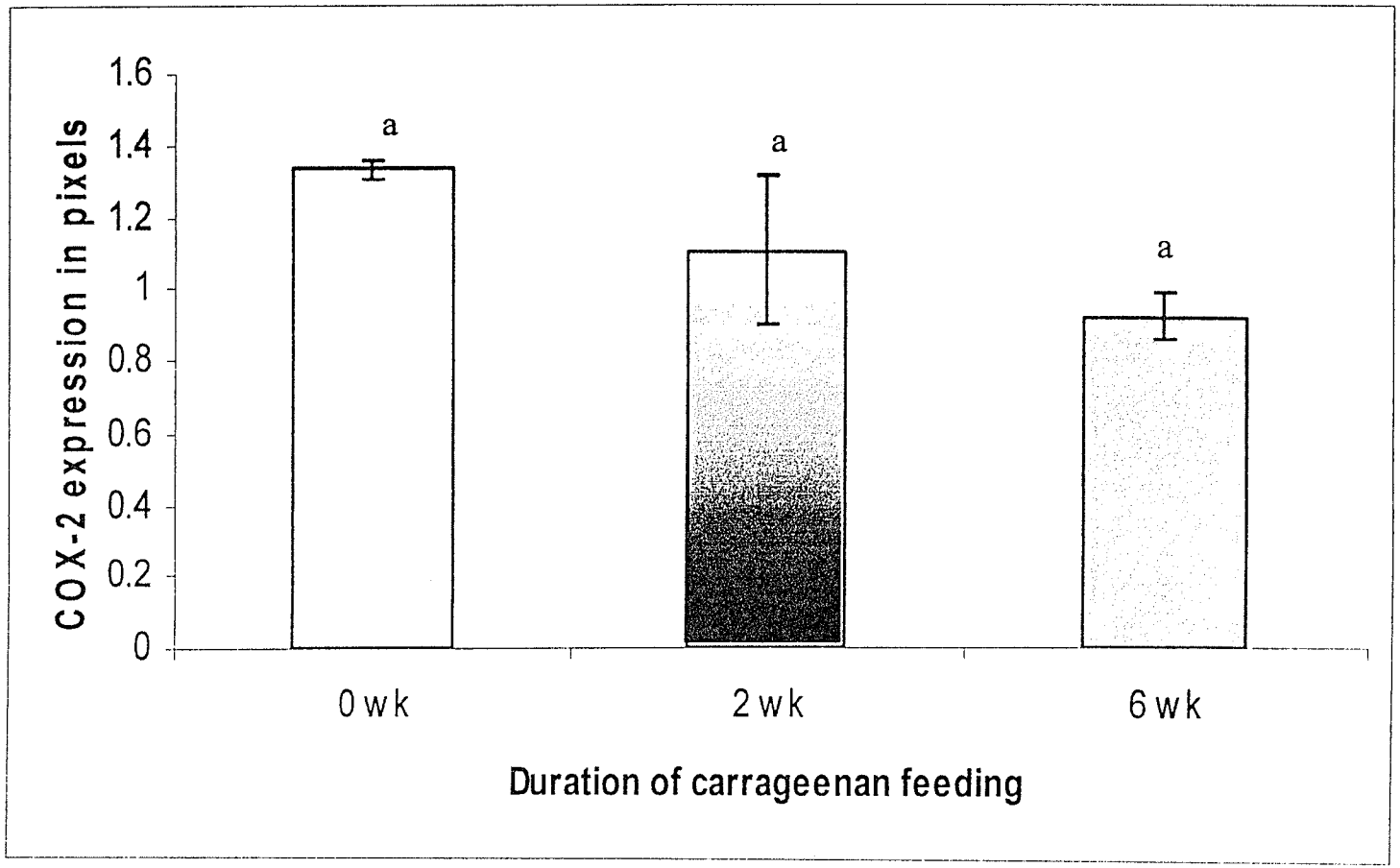

COX-2 expression in pixels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
0 wk
2 wk
6 wk
Duration of carrageenan feeding

Figure 5.7.A TNF-α expression in colonic mucosa as modulated by duration of CAR feeding. Values are means ± SE. Bars labeled with different letters are significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).

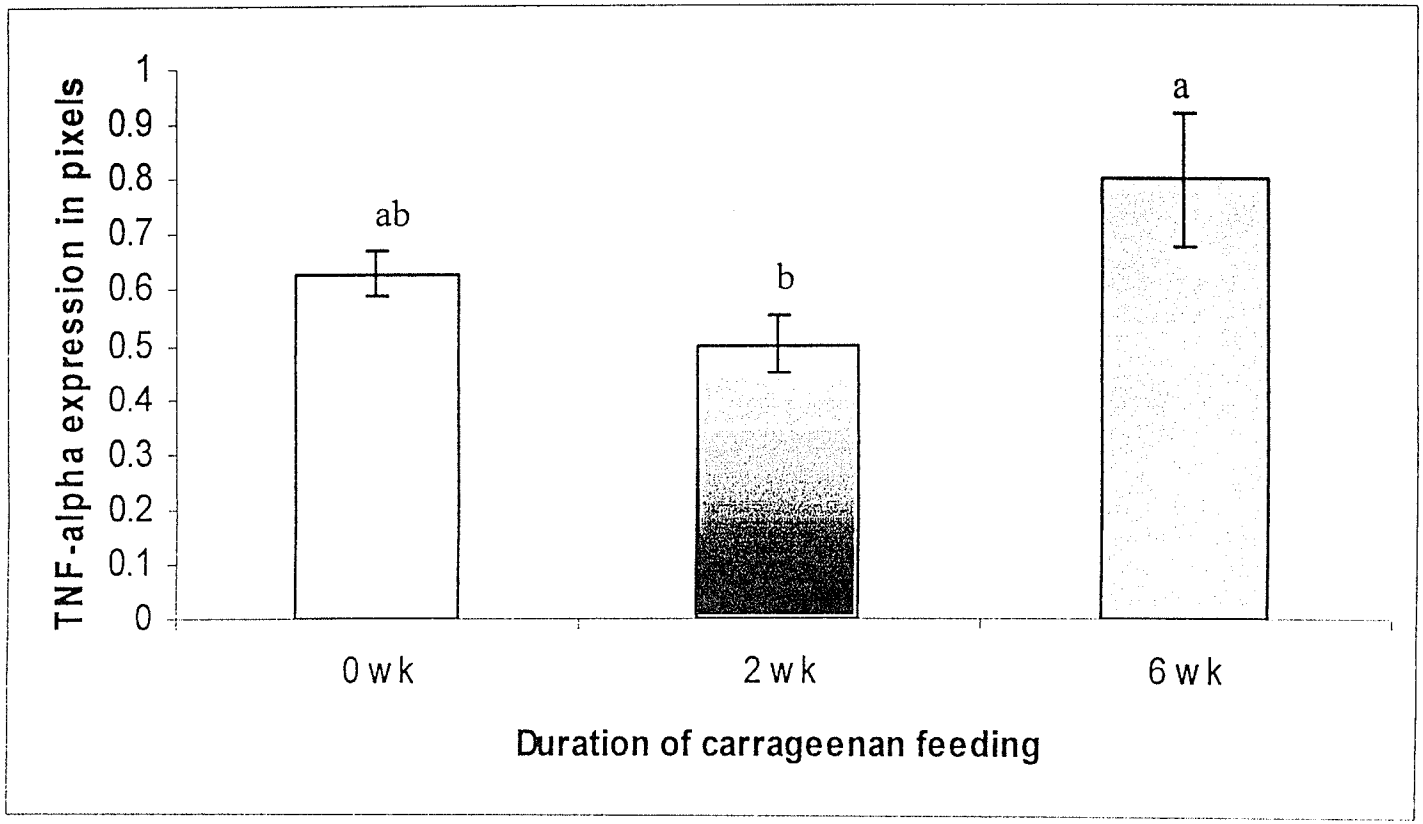
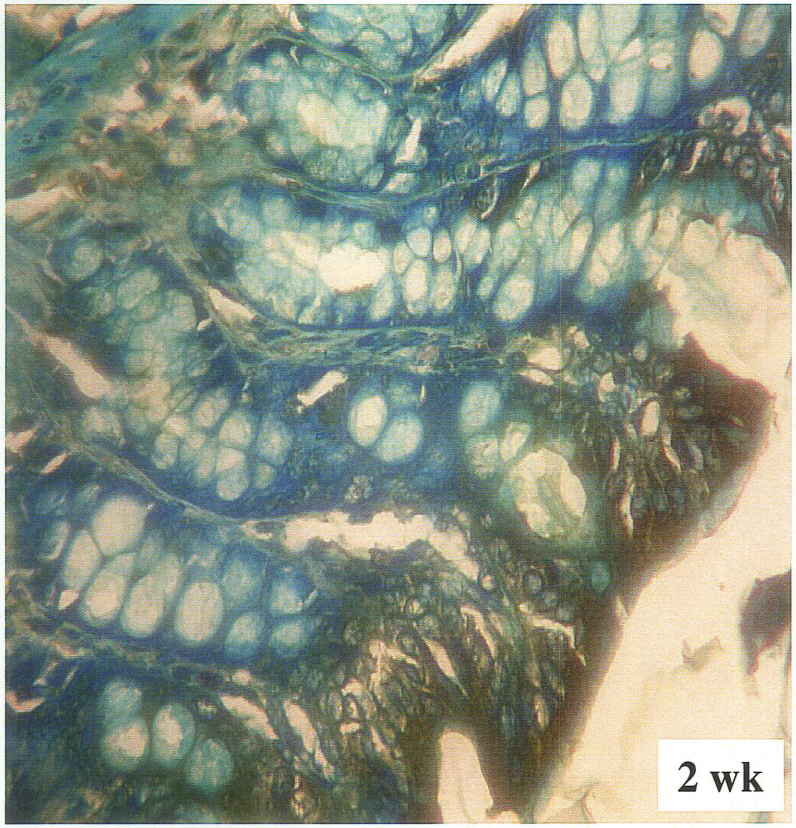

TNF-alpha expression in pixels
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ab
b
a
0 wk
2 wk
6 wk
Duration of carrageenan feeding

Figure 5.7.B Immunohistochemical staining of longitudinal sections of colonic crypts for TNF-α. Magnification x400. Note the marked scattered staining in the lamina propria and colonic submucosa and consistent staining in the apical section of the colonic crypts. 6 wk of CAR feeding resulted in marked increases in the staining intensity in the apical region of colonic crypts, and crypts show epithelial cells crowding. Note more elevated collar like crypt abscess in 6 wk (arrow).


2 wk

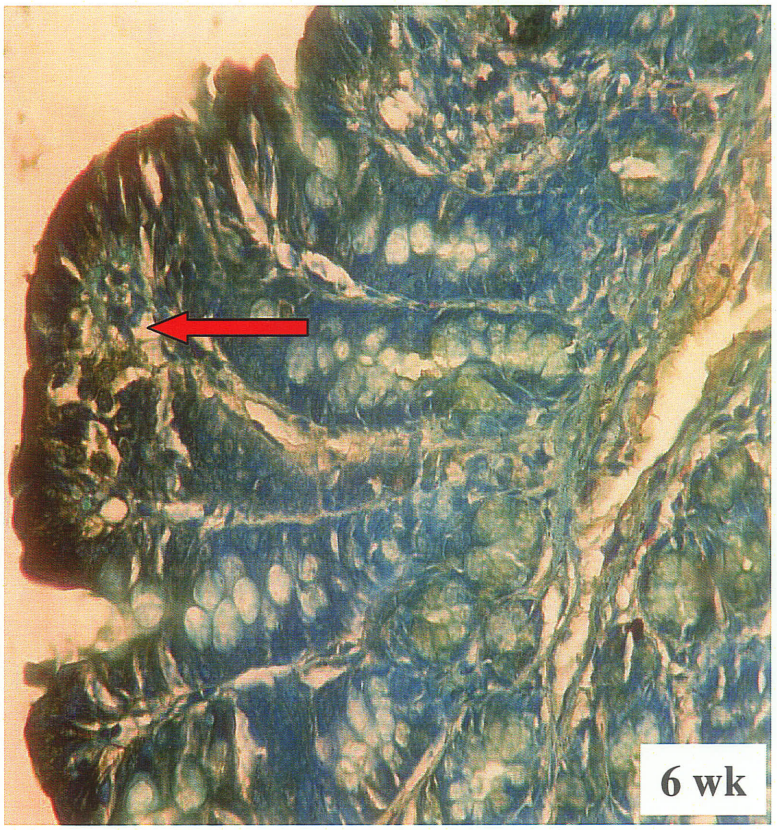

6 wk

Figure 5.8.A iNOS expression in colonic mucosa as modulated by duration of CAR feeding. Values are means ± SE. Bars labeled with different letters are significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).

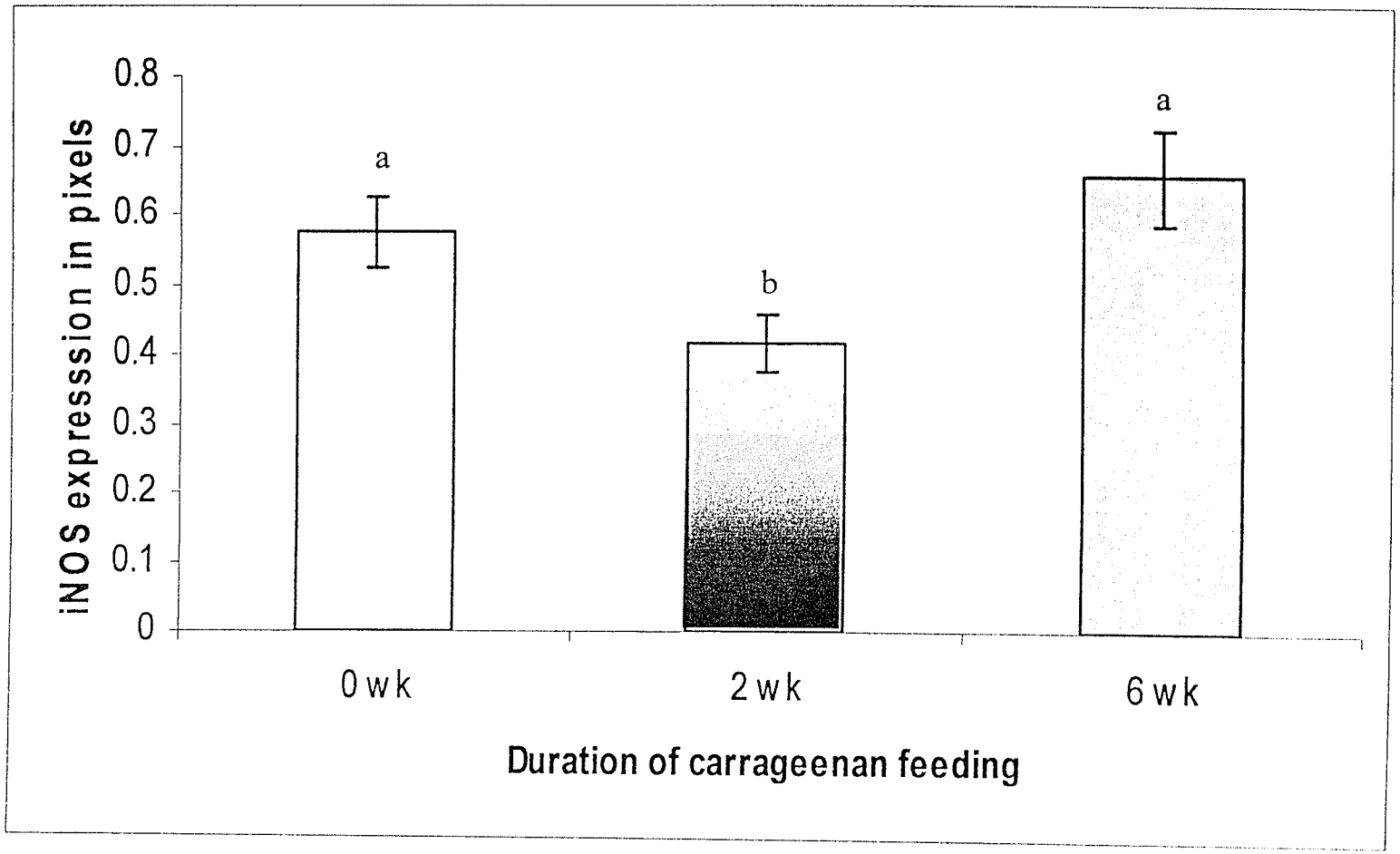

iNOS expresssion in pixels
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
a
0 wk
2 wk
6 wk
Duration of carrageenan feeding

Figure 5.8.B Immunohistochemical staining of longitudinal sections of "swiss rolled" colon for iNOS. Magnification x100. Note brown staining in crypt epithelium, muscularis mucosae, and blood vessels in non-carrageenan treated colon. The staining was consistently absent in colon after 2 wk of carrageenan feeding and then reappeared after 6 wk of feeding.


control

Experiment 2:

A) There were no significant differences between the mean weights of animals treated with CAR for different periods of time and those of control animals. However, animals from the CAR treated groups on average had the lower weights (6 wk group being the lowest) compared to the control group (Table 5.3.). The ACF results (Figure 5.9.) showed that animals treated with LFC+ diet (carrageenan added 2% by weight) for 2, 4 or 6 weeks, injected with AOM and switched to LFC diet had increased number of total ACF as well as the number of ACF with advanced growth features (5 and more crypts/focus) compared to animals from the control group that were fed LFC for the entire duration of the experiment. However, the only group with a significant increase in the total number as well as in the number of ACF with advanced growth features, as compared to the control, was the 6 wk group (Figure 5.10. and Figure 5.11.).

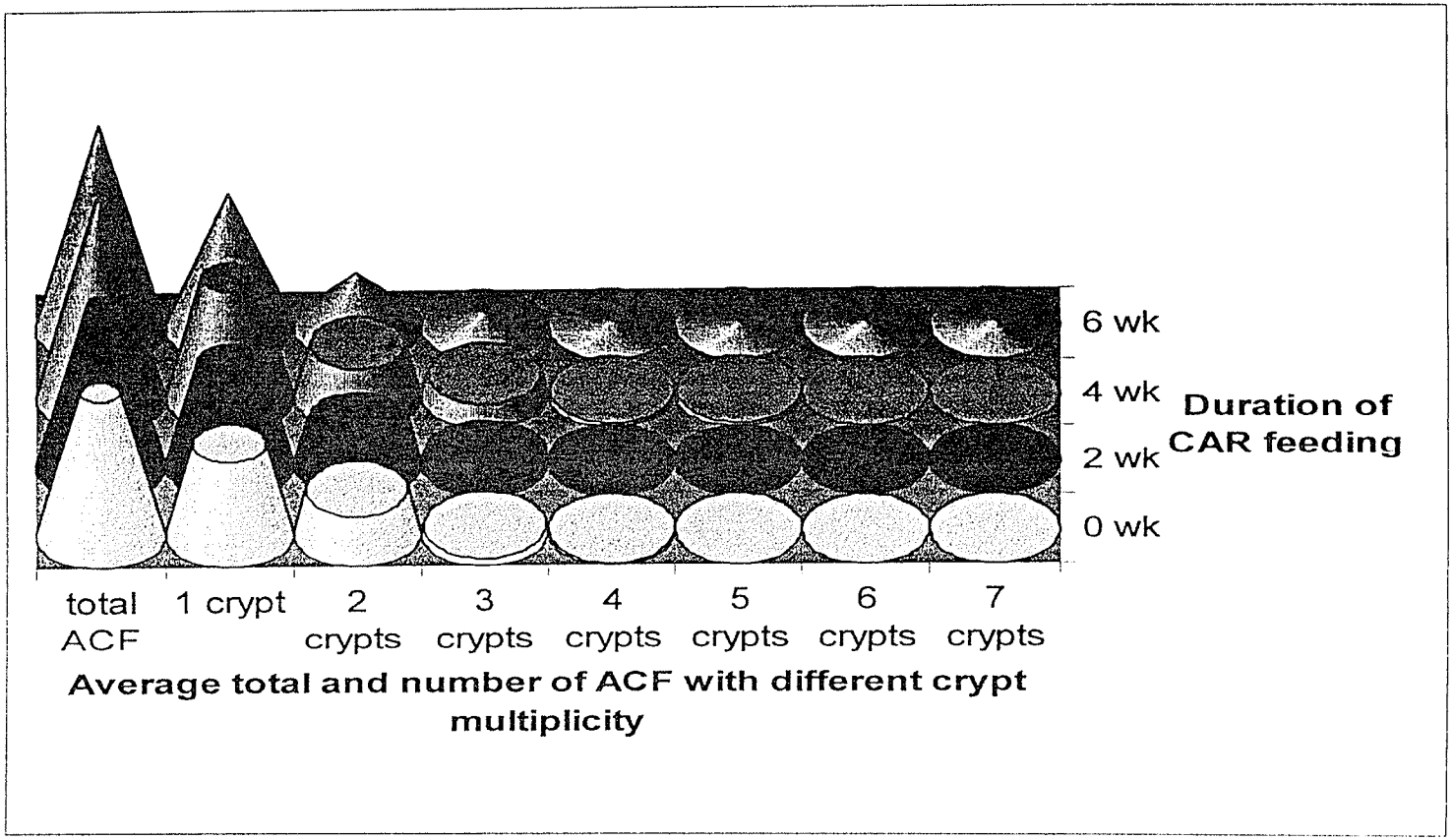
Table 5.3. Average body weights (g) of female Sprague-Dawley rats fed low fat corn oil diet with or without carrageenan for different durations and then injected with AOM.

Experimental groups	Animal weights in grams (Mean ± SE)
control	316 ± 13.7
1 wk	305 ± 40.8
2 wk	301 ± 19.6
4 wk	309 ± 9.1
6 wk	283 ± 12.4

1. Animals (n = 8/group) were fed LFC+ diet for the time periods of 2, 4 and 6 weeks and then injected with AOM and switched to LFC diet. Control animals were fed LFC diet for 6 weeks and then injected with AOM and continued on LFC diet.
2. There was no significant difference among the weights of the animals at $P \leq 0.05$ (ANOVA and Tukey's test).

Figure 5.9. Average total number of ACF and average number of ACF with different crypt multiplicity in female Sprague-Dawley rats fed LFC diet with or without carrageenan for different durations and then injected with AOM.

Crypt multiplicity represents number of crypts/focus. Seven crypts/focus was the largest multiplicity observed during the aberrant crypt foci (ACF) analysis in experiment 2A.



Average total and number of ACF with different crypt multiplicity

Figure 5.10. Total number of ACF in female Sprague-Dawley rats fed LFC diet with or without carrageenan for different durations and then injected with AOM.

Values are means ± SE. Bars labeled with different letters are significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).

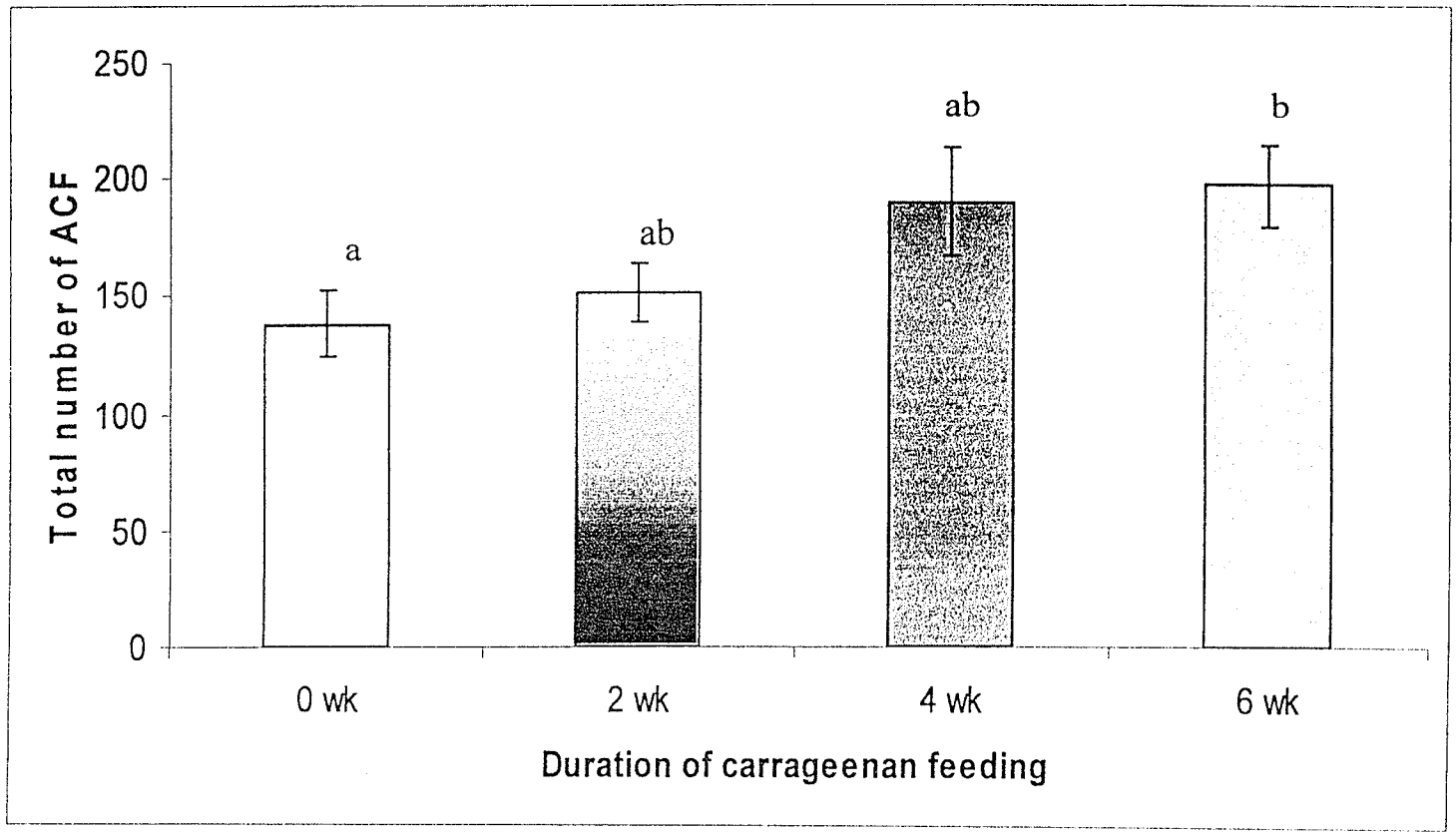
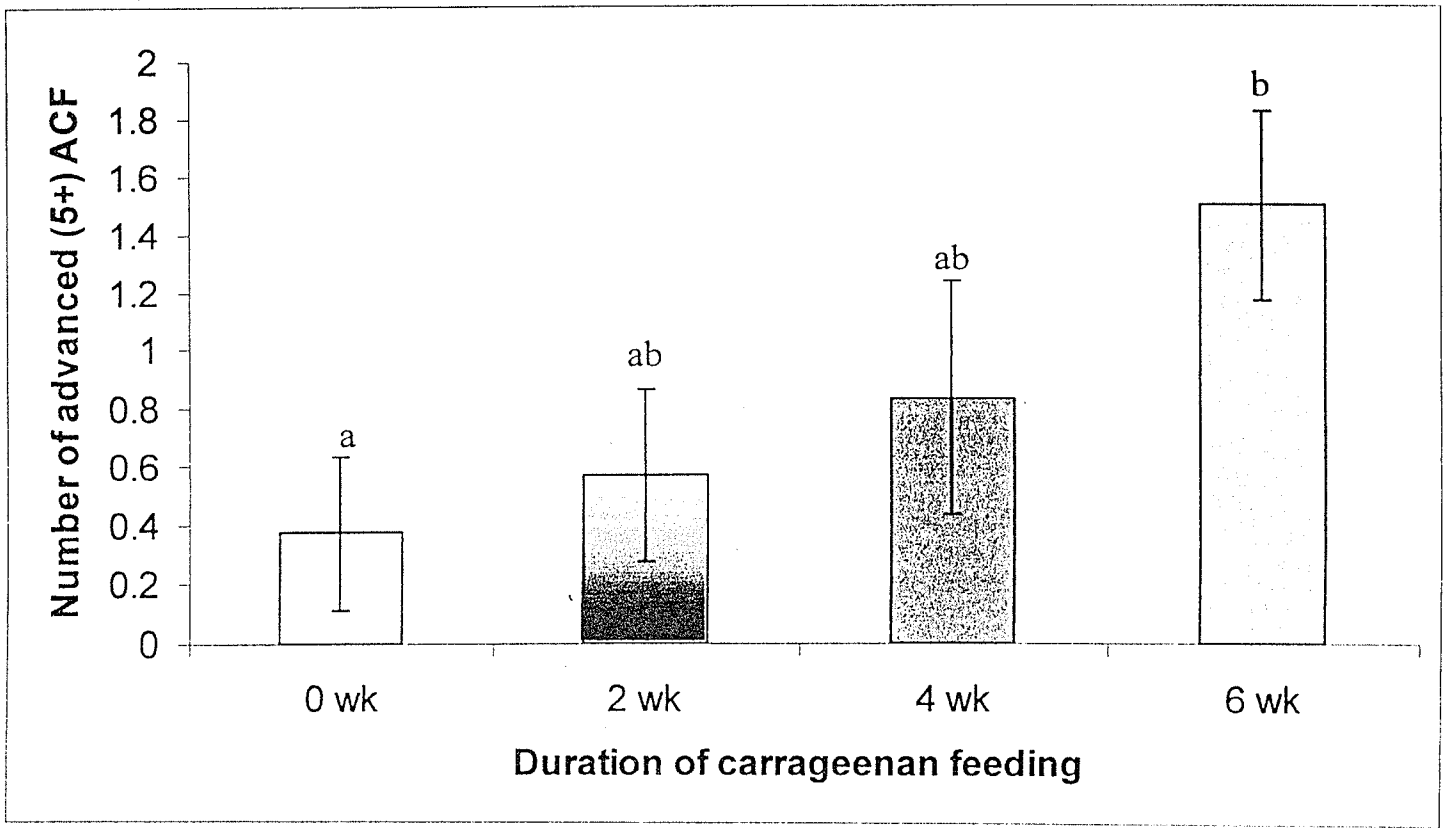

250
200
150
100
50
0
Total number of ACF
a
ab
ab
b
0 wk
2 wk
4 wk
6 wk
Duration of carrageenan feeding

Figure 5.11. The number of advanced (5+) ACF in female Sprague-Dawley rats fed LFC diet with or without carrageenan for different durations and then injected with AOM. Values are means ± SE. Bars labeled with different letters are significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).


Number of advanced (5+) ACF
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
ab
ab
b
0 wk
2 wk
4 wk
6 wk
Duration of carrageenan feeding

B) There were no significant differences in the mean weights of the animals (Table 5.4.) or their major organs (Table 5.5.) at the termination of the Experiment 2B. The ACF analysis showed that animals injected with AOM and then treated with LFC+ diet for 2, 4 or 6 weeks developed significantly higher total number of ACF within 2 weeks of feeding as compared to the control group fed LFC diet during the entire experiment (Figure 5.12.). Moreover, the number of advanced ACF (with 3 or more crypts/focus) significantly increased in the 4 wk and 6 wk groups having approximately 3-fold higher numbers than the animals in control group (Figure 5.13.).

Table 5.4. Average body weights (g) of female Sprague-Dawley rats injected with AOM and concurrently fed with LFC diet with or without carrageenan for different durations.

Experimental groups	Animal weights in grams (Mean ± SE)
control	206.3 ± 13.5
2 wk	211.7 ± 14.5
4 wk	211.7 ± 14.9
6 wk	211.7 ± 13.9

1. Animals (n = 8/group) were injected with AOM and concurrently fed LFC+ diet for the time periods of 2, 4 and 6 weeks. Control animals were fed LFC diet for 6 weeks.
2. There was no significant difference among the weights of the animals at $P \leq 0.05$ (ANOVA and Tukey's test).

Table 5.5. Average organ weights (g) of female Sprague-Dawley rats injected with AOM and concurrently fed with LFC diet with or without carrageenan for different durations.

Experimental groups	Heart weight (Mean ± SE)	Kidney weight (Mean ± SE)	Liver weight (Mean ± SE)	Spleen weight (Mean ± SE)
control	0.94 ± 0.0	2.11 ± 0.1	9.21 ± 0.4	0.56 ± 0.0
2 wk	0.95 ± 0.0	2.20 ± 0.1	9.51 ± 0.5	0.58 ± 0.0
4 wk	0.91 ± 0.0	2.29 ± 0.6	10.15 ± 0.5	0.62 ± 0.0
6 wk	0.92 ± 0.0	2.21 ± 0.1	9.01 ± 0.5	0.55 ± 0.0

1. Animals (n = 8/group) were injected with AOM and concurrently fed LFC+ diet for 2, 4 and 6 weeks. Control animals were fed LFC diet for 6 weeks.
2. There was no significant difference among the weights of the animals' organs at $P \leq 0.05$ (ANOVA and Tukey).

Figure 5.12. Total number of ACF in female Sprague-Dawley rats injected with AOM and concurrently fed LFC diet with or without carrageenan for different durations. Values are means ± SE. Bars labeled with different letters are significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).

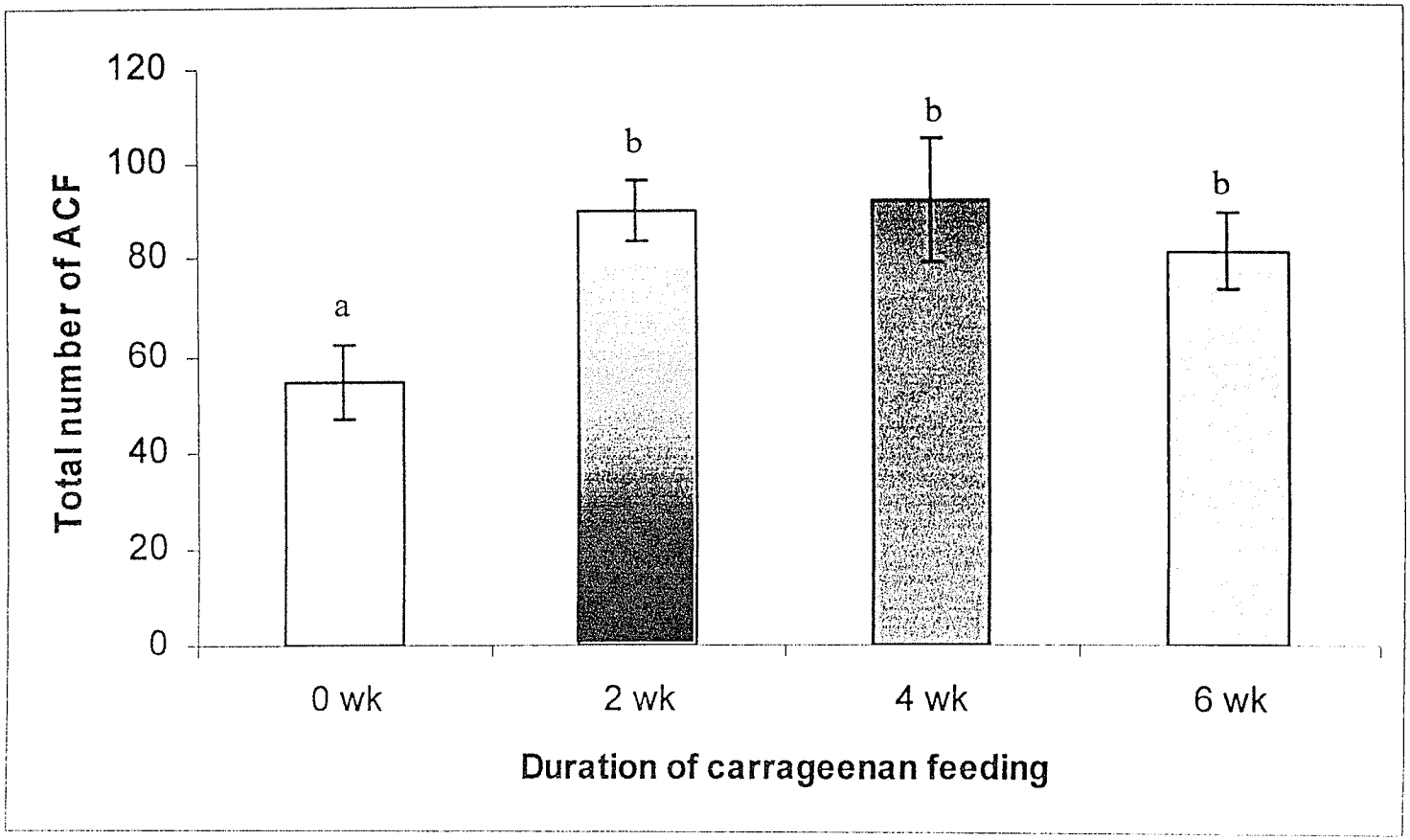
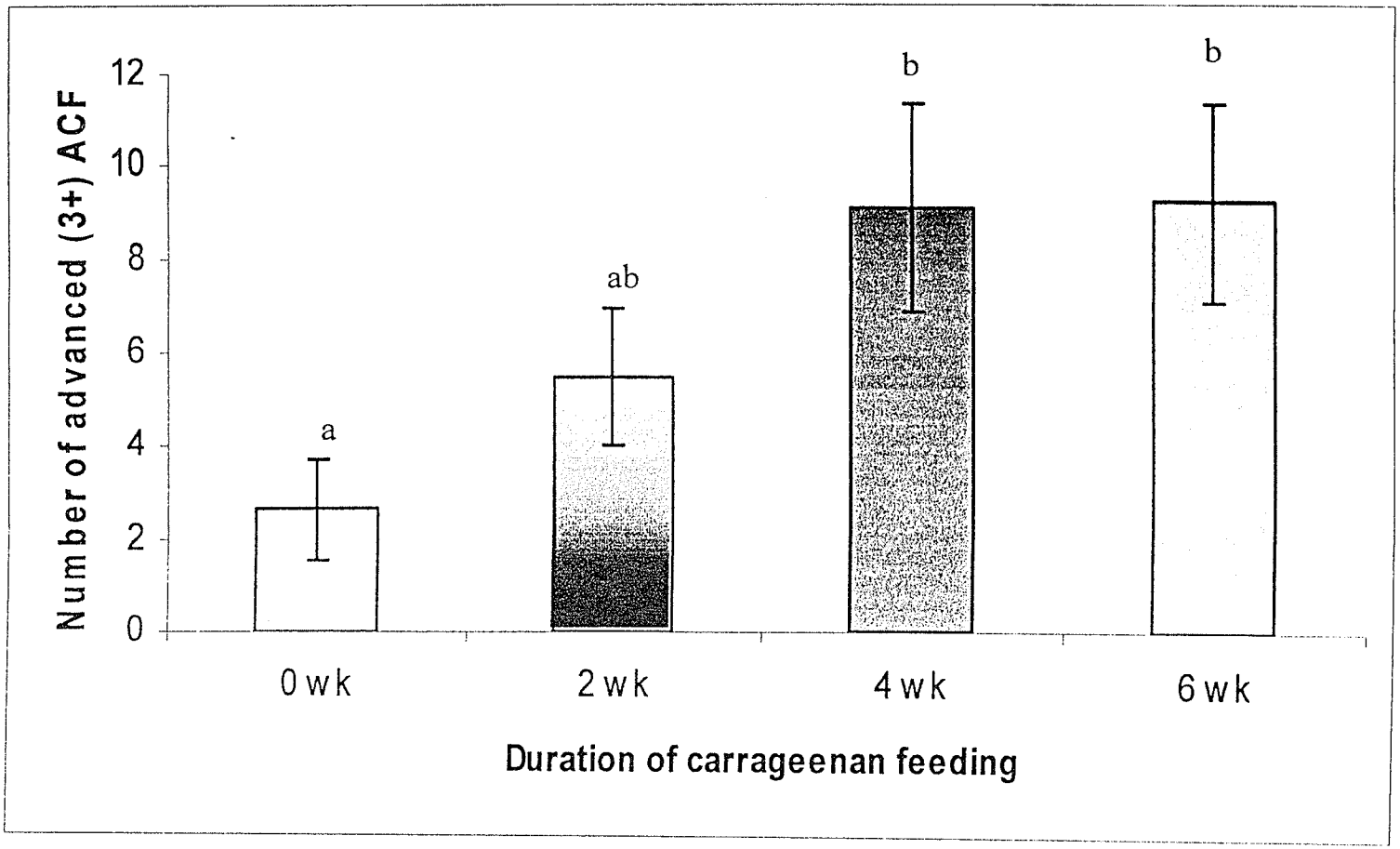

120
100
80
60
40
20
0
Total number of ACF
a
b
b
b
0 wk
2 wk
4 wk
6 wk
Duration of carrageenan feeding

Figure 5.13. The number of advanced (3+) ACF in female Sprague-Dawley rats injected with AOM and concurrently fed LFC diet with or without carrageenan for different durations. Values are means ± SE. Bars labeled with different letters are significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).


Number of advanced (3+) ACF
12
10
8
6
4
2
0
a
ab
b
b
0 wk
2 wk
4 wk
6 wk
Duration of carrageenan feeding

5.3. Discussion

Unlike other colitis-models in which acute inflammation is induced by highly necrogenic chemicals (e.g. acetic acid, formalin, dextran sulphate sodium, or degraded carrageenan), feeding undegraded carrageenan (2% by weight in the diet), proved to be useful in causing moderate and sustained inflammatory changes in colonic mucosa. In a study (Bezabeh et al., 2003) found using magnetic resonance spectroscopy (MRS), that colonic mucosal samples of Sprague-Dawley rats fed LFC+ diet for four days had significantly elevated levels of choline which correlated with the spectrum found in colonic mucosal samples from UC patients by MRS (Bezabeh et al., 2001). First morphological changes that confirmed the presence of inflammation included increased number of lymphocytic infiltration and nodules observed within 1 to 2 weeks of the feeding. By prolonged feeding (4 and 6 weeks) focal erosions and ulcerations characterized with the loss of the surface epithelium and crypts, fibrotic mass and lymphocytes infiltration in the lamina propria were noticed. Animals treated with undegraded CAR prior to or concurrent with AOM injection did not show signs of discomfort, or any visible evidence of distress such as loose stools, blood or mucus in the stool, hair loss or significant weight loss. This indicated that undegraded carrageenan feeding in combination with the specific colon carcinogen, azoxymethane, can be a useful model for investigating development of preneoplastic lesions associated with colitis. Moreover, because there was a low level of discomfort for the involved animals, this model would be very useful in the long-term investigations using tumors as the biological endpoint.

The haematological reaction to CAR feeding pointed to the systematic response

of the body to the undergoing inflammatory changes within colonic mucosa. The fact that platelets (PLTs), neutrophils and monocytes might be involved is a noteworthy observation. PLTs are known to migrate to inflammation site and produce tissue repair cytokines including TGF-β (fibroblast growth factor) (Raju and Bird, 2002). Monocytes are blood cells that migrate into tissues and develop into macrophages where they provide immunological defenses against many antigens. The increase in monocytes observed in this study might be in part explained by the fact that mechanism of CAR-induced inflammation involves uptake of CAR by intestinal macrophages, followed by leakage of lysosomal enzymes into the mucosal tissue and consequent tissue destruction and inflammation (Kim and Berstad, 1992; Strober, 1985; Fabian et al., 1973). Neutrophils are polymorphonuclear white blood cells that respond to chemotactic signals produced during inflammatory process. They are concentrated in inflammation area and are rapidly used, leaving less circulating cells in the blood (Cotran et al., 1999). Thus the decrease in the number of neutrophils in the blood during the first two weeks of CAR feeding could be due to their migration to the colonic tissue. The possible involvement of PLTs, monocytes and neutrophils in CAR-caused colitis needs to be confirmed in the future studies. It would be important to determine, using a large number of animals, if the systemic changes noted in the present study would also occur with longer LFC+ feeding or due to inflammation induced by other agents.

At the molecular level, duration of CAR feeding did influence expressions of molecules known to play a role in the inflammatory process as well as in carcinogenesis. TNF-α, one of the most potent pro-inflammatory cytokines first decreased (2 wk of CAR feeding) and then increased with the duration of inflammation. The same trend was

observed with the iNOS. This increase of TNF-α and iNOS could in part explain increased sustained inflammatory changes as well as the higher sensitivity of colonic mucosa to ACF development after 6 weeks of CAR feeding. TNF-α involvement in the mediation of inflammatory process is either direct, for example by chemoattracting neutrophils and monocytes (Bischoff et al., 1999; Cotran et al., 1999), or indirect by inducing other pro-inflammatory cytokines, growth factors, and pro-inflammatory enzymes including iNOS (Marion, et al., 2003; Colon et al., 2001) and COX-2 (Jobin et al., 1998; Yamamoto et al., 1995). The elevated levels of TNF-α as well as iNOS and its product nitric oxide (NO) have been reported in inflamed mucosa of patients with UC (Kankuri et al., 2003; Dijkstra et al., 1998). iNOS over-expression has also been demonstrated in several animal models of IBD (Colon et al., 2001; Southey et al., 1997). When over-expressed, NO and its metabolites such as nitrogen dioxide and peroxynitrite, can have damaging effects on proteins and DNA structure that could contribute to gene mutations and development of carcinoma. One of the limitations of this study was that activity of the targeted molecules was not assessed. Moreover, the reason for the decline of the TNF-α and iNOS after 2 weeks of CAR feeding followed by their increase after 6 weeks of feeding cannot be explained at this point. Our speculation is that during different phases of pathogenesis these molecules have different roles, as documented in other studies (for review see 2.1.2.0.).

There is increasing evidence that COX-2 plays a major role in mediation of both pathological processes: inflammation and carcinogenesis. Human and animal studies have demonstrated an over-expression of COX-2 and its products (PGE2, PGI2 and TxA2) in UC (Singer et al., 1998; Reuter et al., 1996; Raab et al.,1995; De Dombal et al.,

1993) as well as in colonic adenomas and carcinomas (Shacter and Weitzman, 2002; Shattuck-Brandt et al., 2000; Kawamori et al., 1998; DuBois, et al., 1996; Eberhart et al., 1994). A recent study by Hill and colleagues (2004) is one of the rare investigations done on the involvement of COX-2 in UC-CRC development. The authors compared carcinomas arising in inflammatory bowel disease versus sporadic carcinomas and found significantly increased expression of COX-2 in colitis-associated carcinomas. In the present study levels of COX-2 in the colonic mucosa declined as the duration of feeding with CAR increased. ACF results showed that decreased COX-2 status of colonic mucosa had increased sensitivity to the development of preneoplastic lesions. We speculate that basal level of COX-2 in colonic mucosa might have protective role, and a decreased level renders it more vulnerable to carcinogenesis. Reuter and colleagues (1996), reported exacerbation followed by colonic perforation after administration of COX-2 selective inhibitors to rats with chemically induced colitis suggesting its protective role in the UC. Overall, it seems that the inflammatory state represented by decreased COX-2 and increased TNF-α and iNOS levels in colonic mucosa make it more sensitive to colon carcinogenesis. However, whether or not they have a role in UC-associated carcinogenesis remains to be seen.

Results from Experiment 2A showed that a longer duration of inflammation before injection of carcinogen gradually increased sensitivity of colonic mucosa to preneoplastic development; the 6 wk group, with the longest duration of inflammation in this study, had significantly higher total number of ACF and higher proportion of animals with advanced ACF growth features compared to the control. This finding agrees with epidemiological studies reporting that duration of inflammation plays a critical role in the

development of colonic adenocarcinomas in ulcerative colitis patients (Eaden et al., 2001; Cooper et al., 2000; Tsianos, 2000; Bernstein et al., 1994), as well as with reports showing that UC patients face up to a sevenfold increased risk for colorectal cancer development compared to the normal population (Shacter and Wietzman, 2002; Eaden et al., 2001; Pohl et al., 2000).

In Experiment 2B we wanted to determine if duration of inflammation initiated concurrently with carcinogen feeding would alter the risk of developing colon cancer. The results showed that even within 2 weeks of CAR feeding cocarcinogenic effect was demonstrated by significant increase in the number of the ACF as compared to the control. This experiment also demonstrated that the longer the duration of inflammation administrated concurrently with a carcinogen significantly promoted development of advanced preneoplastic lesions as seen in 4 and 6 wk groups. Combining the ACF results from the both experiments performed in this study, it can be concluded that the inflammatory process present before and concurrent with carcinogen injection is cocarcinogenic and that colonic mucosa response differs before or after the injection.

To our knowledge, this is the first study, which experimentally has demonstrated that duration of inflammation has significant effect on the risk for developing colon cancer. The findings of this study while being informative, cautions need to be taken because this was a short-term model. These findings need to be confirmed with longer studies using tumors as biological endpoint.

5.4. Summary

This study demonstrated that CAR induces inflammatory responses detectable morphologically by 2 to 6 weeks. CAR feeding (duration) enhances the sensitivity of colon to AOM induced carcinogenesis. This increased sensitivity might be due in part to decreased COX-2 and increased iNOS and TNF-α levels in colonic mucosa. Inflammation prior to AOM injection is cocarcinogenic and duration of inflammation may play a key role in enhancing colonic cancer development. CAR feeding concurrent with AOM injection accelerated ACF number and growth. Feeding CAR in the diet as a post-carcinogen treatment may not be crucial for tumor development.

CHAPTER 6

Modulation of precancerous lesions and protein expression of iNOS and COX-2 in colonic mucosae by the amount and type of dietary lipids in carrageenan model of ulcerative colitis

6.0. Introduction

Several epidemiological and case-control studies demonstrated a positive association between consumption of fat, especially fat of animal origin, and increase in incidence of colon cancer (Giovannucci and Willett, 1994; Hursting et al., 1990; Willett et al., 1990; Potter and McMichael, 1986). However, there have been no studies investigating the modulating effect of dietary lipids on development of colon cancer under inflammatory conditions. It is hypothesized that dietary intervention with a high flaxseed oil diet, due to its high content of α-linolenic acid (18:3 ω-3), will reduce inflammation, which will, in turn, lower the heightened risk of developing colon cancer, whereas a high corn oil diet rich in linolenic acid (18:2 ω-6) will not. This hypothesis was tested in our laboratory in a preliminary study (Dahleh, 2001).

The results from the preliminary study showed that in the absence of inflammation high fat ω-6 diet promotes ACF development while high ω-3 as well as low LFC have a protective effect. In the presence of inflammation, however, both high fat diets (HFC+ ω -6 and HFF+ ω -3) reduced the growth and number of ACF, while low fat LFC+ ω-6 diet promoted it. The most noteworthy observation was that in inflamed colons high fat ω -6 diet, which normally (in the absence of inflammation) promotes the growth of ACF or tumors, acted in an opposite manner similar to a high fat ω -3 diet in reducing the number of ACF. These findings supported the potential of dietary lipids to serve as

preventive agents in reducing the associated risk of developing colon cancer in UC patients. However, these findings also raised an important question, that is, is it high fat diet per se or the type of fat (PUFAs) that may effect colitis-associated carcinogenesis? Because CAR had no effect in high fat ω-6 and ω-3 fed groups we wondered if a similar outcome will be noted in other types of high fat diet, such as olive oil and beef tallow that are rich sources of oleic acid or stearic acid, respectively.

Based on our hypothesis that a high fat diet per se might reduce the risk of colon cancer development in the CAR induced model of ulcerative colitis, the main objectives of the present study were:

1. To understand the effect of high fat diets, varying in fatty acid composition, on the ability of CAR to augment the formation of colonic preneoplastic lesions.
2. To assess whether high and low content of ω-6 PUFAs in a diet affects the levels of pro-inflammatory molecules in carcinogen treated animals differently in the presence of CAR.

6.1. Materials and methods

Female, Sprague-Dawley rats (Charles River, Montreal, Canada) weighing approximately 75-80g, were acclimatized for 1 week on standard rat chow. Animals were housed, two to three per cage, in wire meshed stainless steel cages with sawdust bedding in a room under controlled environmental conditions (21°C and 50% humidity) and photoperiod (12 hr light/dark cycle). All procedures involving the animals were approved by the University of Manitoba Animal Care Committee and were conducted according to the guidelines of the Canadian Council of Animal Care. Diets were based on modified

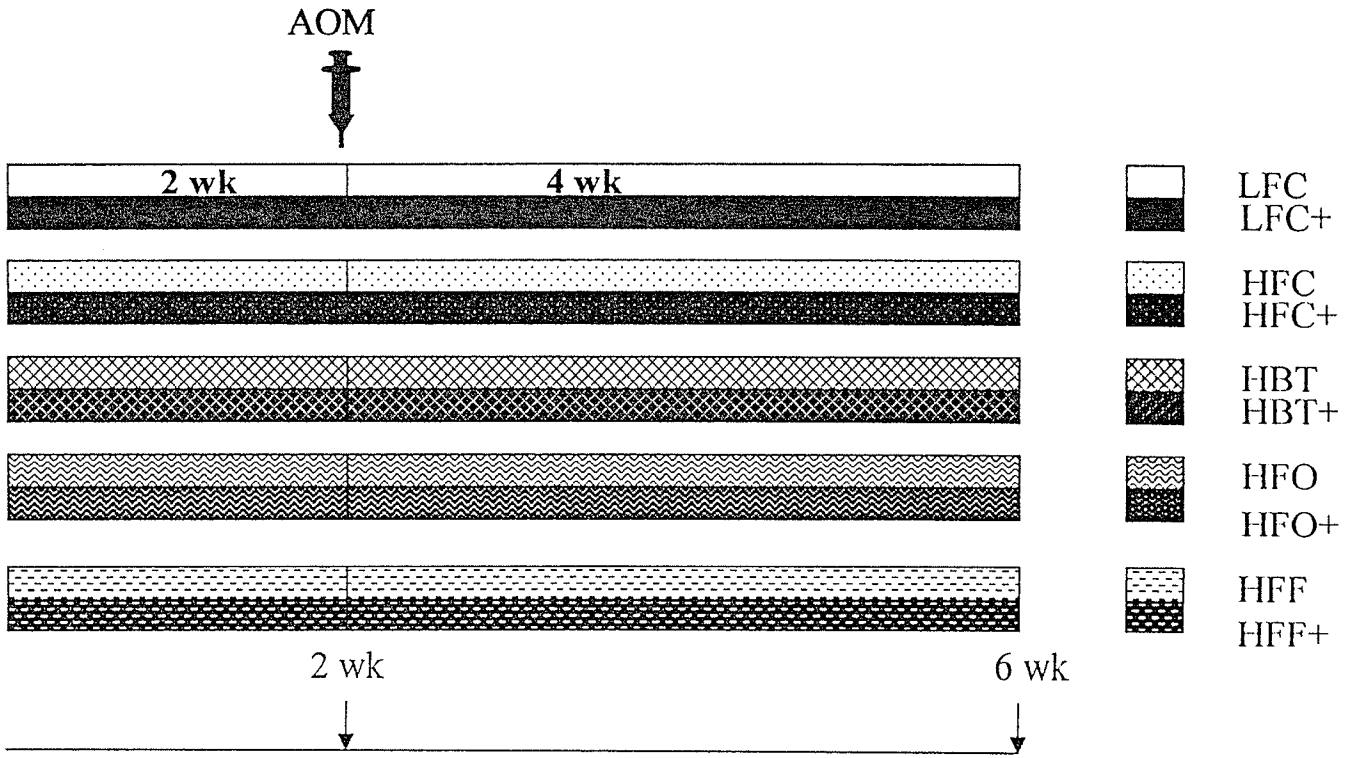
AIN-93 diet: low fat corn oil diet (LFC) contained 5% corn oil, high fat corn oil diet (HFC) contained 23.8% corn oil, high fat olive oil and beef tallow diets contained 5% corn oil + 18.8% test oil, and high fat flaxseed oil diet contained 14.3% corn oil and 9.5% flaxseed oil. Undegraded Commercial Type I Carrageenan (Sigma Chemical Co.) was added to the diet (2% by weight). Animals had access to food and water *ad libitum*.

The fatty acid composition of experimental diets was determined by GC. All materials and techniques used are described in detail in Chapter 4.

6.1.0. Study design

See Figure 6.1. for the schematic representation of the experimental design. After a period of acclimatization rats were randomly allocated into ten groups and fed one of the following diets for 6 weeks: low fat corn oil diet (LFC) without carrageenan or with carrageenan (LFC+), high fat corn oil diet (HFC) without or with carrageenan (HFC+), high fat beef tallow diet (HFB) without or with carrageenan (HFB+), high fat olive oil diet (HFO) without or with carrageenan (HFO+), and high fat flaxseed oil diet (HFF) without or with carrageenan (HFF+). After two weeks of feeding all animals were injected with AOM (10 mg/ kg body wt). Food intake, initial and weekly body weights were recorded. At the end of 6 weeks, all animals were terminated and their colons used either for ACF scoring (8 animals per group) or assessment of inflammatory changes (LFC, LFC+, HFC and HFC+ /5 animals per group).

Figure 6.1. Schematic representation of the experimental design



Low fat corn oil diet = LFC
High fat corn oil diet = HFC
High fat beef tallow diet = HFB
High fat olive oil diet = HFO
High fat flaxseed oil diet = HFF
(+) sign represents diet with carrageenan
Azoxymethane = AOM

6.2. Results

The fatty acids composition of dietary lipids employed in the study was as expected (Tables 6.1. and 6.2.), that is, corn oil diets (LFC and HFC) had a high content of polyunsaturated fatty acids (PUFA) mostly 18:2 ω-6 FA, olive oil diet was rich in monounsaturated fatty acids (MUFA) mostly 18:1ω-9, flaxseed oil diet was rich in PUFAs 18:2 ω-6 and 18:3 ω-3 (ratio 2:3), and beef tallow oil diet was rich in saturated fats mostly 16:0 and 18:0.

The change in stool consistency or presence of blood or mucus in the stool was not observed. The mean daily food intake showed that in general animals received diets with CAR had slightly higher daily intake than those animals received diets without CAR (Table 6.3.). However, even though the intake of food was slightly higher among the animals fed diets with CAR, at the end of the study the animals fed diets without CAR had slightly higher mean weights than their CAR fed counterparts. Overall, there was no significant difference in animal weights across different dietary groups regardless of type and amount of fat and carrageenan feeding (Table 6.4.).

ACF analysis showed that compared to LFC, high fat diets (HFB, HFC and HFO) promoted the number (Figure 6.2.) and growth (Figure 6.3.) of ACF with the exception of HFF diet.

Table 6.1. Fatty acid composition of corn oil, flaxseed oil, olive oil, and beef tallow based diets (%)

Fatty acid	Corn oil diet	Flaxseed oil diet	Olive oil diet	Beef tallow diet
C 10: 0				0.066
C 12: 0				0.067
C 14: 0	0.041	0.044	0.036	1.882
C 14: 1				0.268
C 15: 0		0.02		0.292
C 16: 0	10.817	8.482	13.117	21.830
C 16: 1	0.111	0.091	1.029	1.833
C 17: 0	0.084	0.082	0.082	0.945
C 18: 0	1.872	2.693	2.592	16.995
C 18: 1ω9	29.131	25.295	58.672	34.182
C 18: 1ω11	0.584	0.577	2.015	1.420
C 18: 2ω6	55.282	39.259	20.643	18.343
C 18: 3ω6				0.029
C 20: 0	0.491	0.434	0.537	0.336
C 18: 3ω3	0.958	22.401	0.650	
C 20: 1	0.278	0.240	0.298	
C 20: 2	0.039	0.036		0.177
C 20:3ω4				0.067
C 22: 0	0.165	0.152	0.168	0.075
C 20: 3ω3		0.026		0.028
C 20: 4				0.105
C 22:2			0.027	
C 22: 4				0.057
C 22: 5				0.050
C 22: 6			0.021	
C 24: 0	0.199	0.163	0.103	0.505

Table 6.2. Percentage of saturated, MUFA and PUFA fatty acids in corn oil, flaxseed oil, olive oil and beef tallow based diets.

Fatty acid	Corn oil diet	Flaxseed oil diet	Olive oil diet	Beef tallow diet
Saturated	13.665	12.060	16.635	42.535
MUFA	30.108	26.205	62.010	38.025
PUFA	56.279	61.733	21.355	19.440
18:2ω-6*	55.280	39.260	20.643	18.340
18:8 ω-3*	0.958	22.401	0.650	/

1. Values are expressed as percentages.
2. Abbreviations are as follows: MUFA (monounsaturated fatty acids); PUFA (polyunsaturated fatty acids).
3. * PUFAs shown as percentage of ω-6 and ω-3 in each experimental diet.

Table 6.3. Mean daily intake values for female Sprague-Dawley rats fed one of five diets: LFC, HFC, HFF, HFO or HBT with (+) or without addition of carrageenan 2 weeks prior and 4 weeks after AOM injection

Experimental diets	Mean daily intake (g/day/animal)
LFC	11.9 ± 0.6
LFC+	11.5 ± 0.4
HFC	9.9 ± 0.6
HFC+	10.6 ± 0.1
HBT	10.1 ± 0.2
HBT+	10.7 ± 0.7
HFO	8.8 ± 0.7
HFO+	9.4 ± 1.4
HFF	10.2 ± 0.4
HFF+	11.2 ± 0.5

1. Values are mean ± SE ($n \geq 8$ animals/group).
2. Abbreviations are as follows: LFC (low fat corn oil diet); HFC (high fat corn oil diet); HFF (high fat flaxseed oil diet); HFO (high fat olive oil diet); HBT (high fat beef tallow diet) without or with (+) addition of carrageenan
3. There was no significant difference in mean daily intake of food among the rats belonging to different experimental dietary groups ($P < 0.05$).

Table 6.4. Average body weights of for female Sprague-Dawley rats fed one of five diets: LFC, HFC, HFF, HFO or HBT with (+) or without carrageenan 2 weeks priorand 4 weeks after AOM injection

Experimental diets	Animal weights (g) (Mean ± SE)
LFC	252.5 ± 10.3
LFC+	237.9 ± 7.5
HFC	266.7 ± 8.5
HFC+	264.8 ± 11.9
HBT	258.8 ± 9.6
HBT+	245.8 ± 10.1
HFO	256.5 ± 6.3
HFO+	238.5 ± 4.9
HFF	258.7 ± 11.2
HFF+	240.9 ± 7.0

1. Values are mean ± SE ($n \geq 8$ animals/group).
2. Abbreviations are as follows: LFC (low fat corn oil diet); HFC (high fat corn oil diet); HFF (high fat flaxseed oil diet); HFO (high fat olive oil diet); HBT (high fat beef tallow diet) without or with (+) addition of carrageenan
3. There was no significant difference in mean daily intake of food among the rats belonging to different experimental dietary groups ($P < 0.05$).

Figure 6.2. Total number of ACF in female Sprague-Dawley rats fed one of five diets: LFC, HFC, HFB, HFO or HFF, 2 weeks prior and 4 weeks after AOM injection. Values are means ± SE. Bars labeled with different letters are significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).

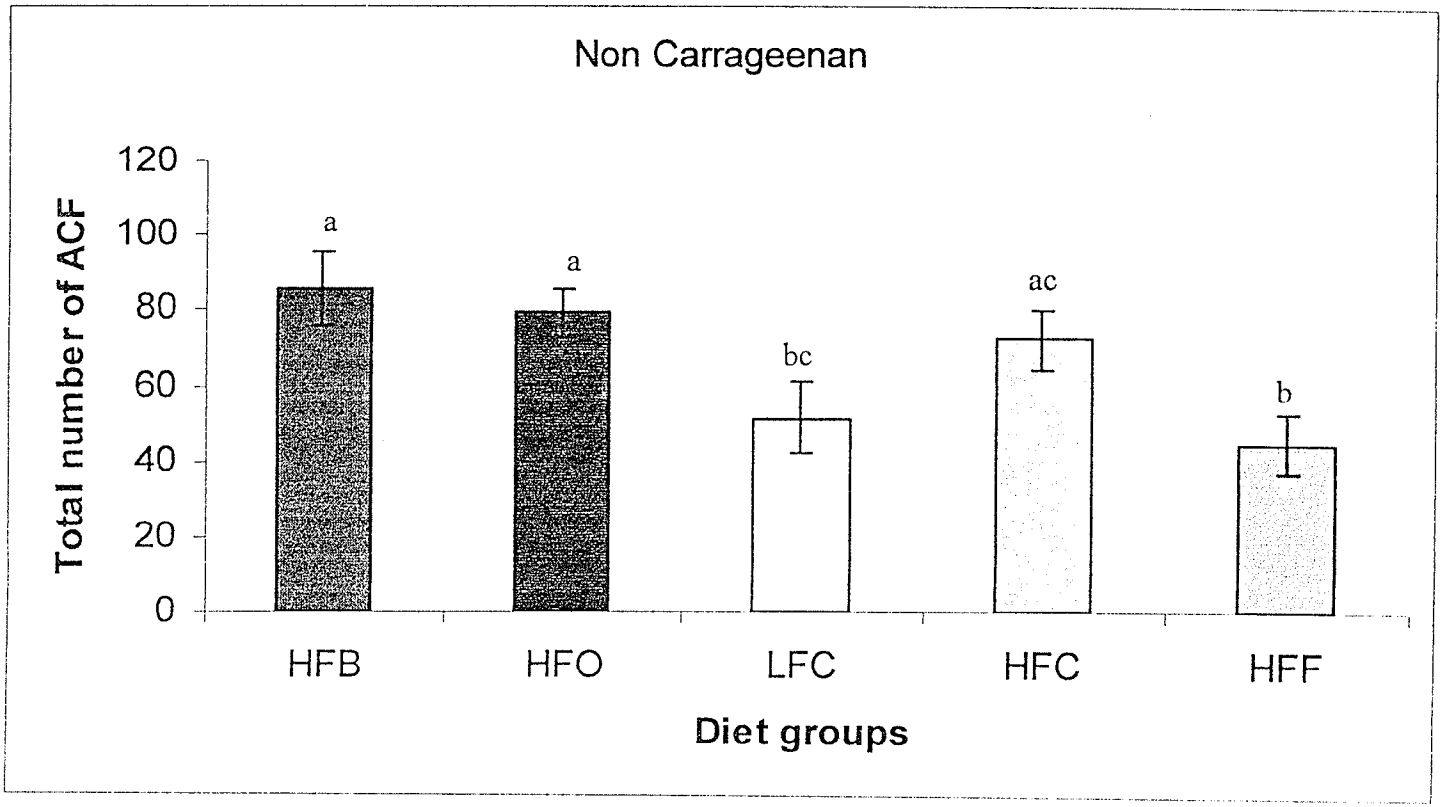
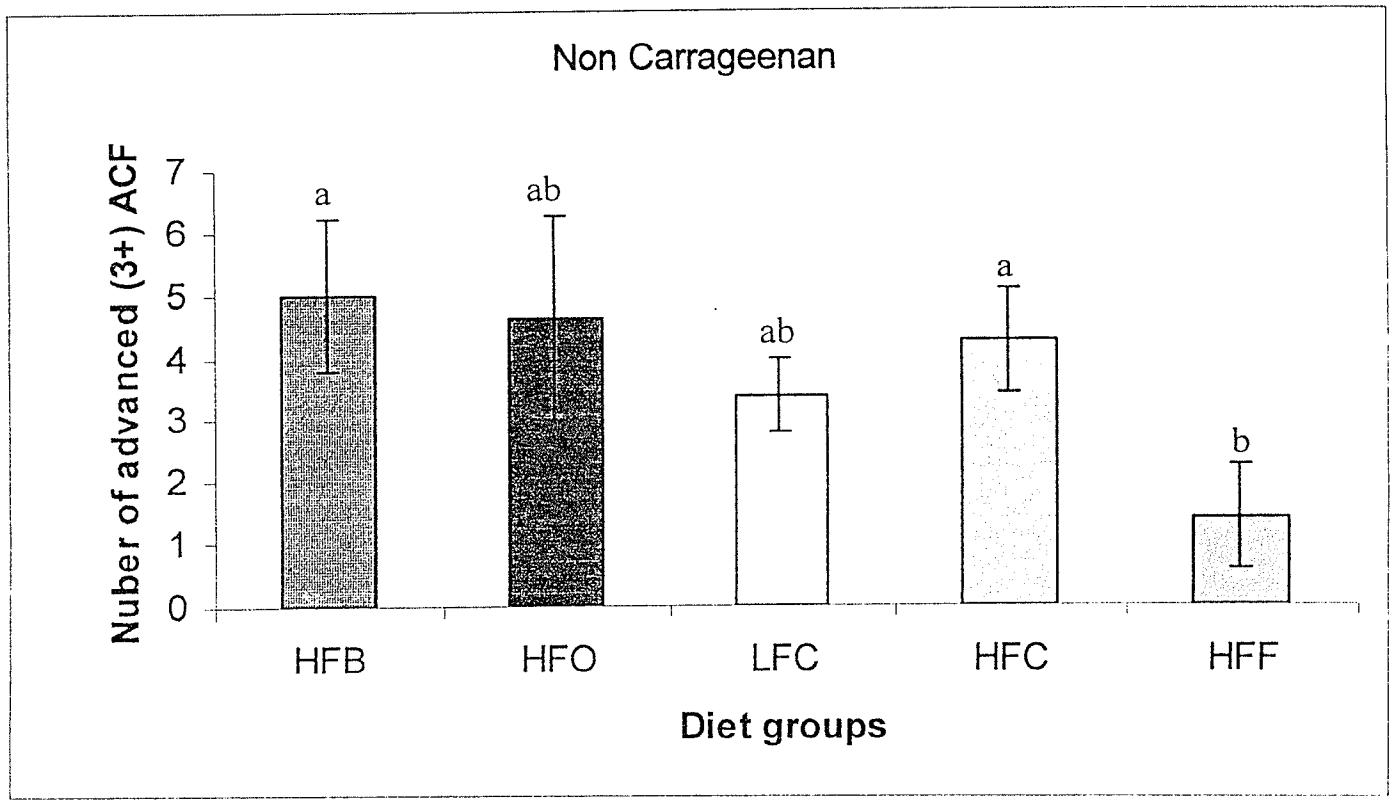

Non Carrageenan
Total number of ACF
120
100
80
60
40
20
0
a
a
bc
ac
b
HFB
HFO
LFC
HFC
HFF
Diet groups

Figure 6.3. Number of advanced (3+) ACF in female Sprague-Dawley rats fed one of five diets: LFC, HFC, HFB, HFO or HFF, 2 weeks prior and 4 weeks after AOM injection. Values are means ± SE. Bars labeled with different letters are significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).


Non Carrageenan
Nuber of advanced (3+) ACF
0
1
2
3
4
5
6
7
a
ab
ab
a
b
HFB
HFO
LFC
HFC
HFF
Diet groups

Among high fat diets without CAR, HFB diet had the strongest promotional effect with the highest number of total as well as advanced ACF (Figure 6.4.). Among all CAR diets, only LFC+ diet promoted the number of ACF, while HFC+ diet significantly reduced the number of ACF compared to other CAR diets (Figure 6.5.). Even though animals fed HFC+ diet had the overall lowest total number of ACF of all groups, animals fed HFF or HFF+ diet had the lowest number of advanced ACF (3 and more crypts/focus) as shown in Table 6.5.

In an attempt to explain the differences in the colonic mucosa sensitivity to ACF development in animals fed different amount of corn oil diet with or without CAR (observed in the preliminary study), four groups LFC, LFC+, HFC, and HFC+ were selected to determine if their colonic mucosa would differ from each other biochemically. Therefore, Western blot analysis was performed on colonic mucosa of animals treated with LFC and HFC diets with or without carrageenan. The results showed that the inflammatory molecules COX-2 and iNOS, observed as protein bands 72kDa and 130kDa respectively, exerted the same trend, that is, animals fed LFC diet had less COX-2 and iNOS expressed in their colonic mucosa, compared to the LFC+ group. In contrast, animals fed HFC diet had more COX-2 and iNOS levels in their colonic mucosa than their counterparts fed HFC+ diet (Figure 6.6. and Figure 6.7.).

Figure 6.4. Average total number of ACF and average number of ACF with different crypt multiplicity in female Sprague-Dawley rats fed one five diets: LFC, HFC, HFB, HFO or HFF, 2 weeks prior and 4 weeks after AOM injection.

Crypt multiplicity represents number of crypts/focus. Five crypts/focus was the largest multiplicity observed during the aberrant crypt foci (ACF) analysis in this experiment.

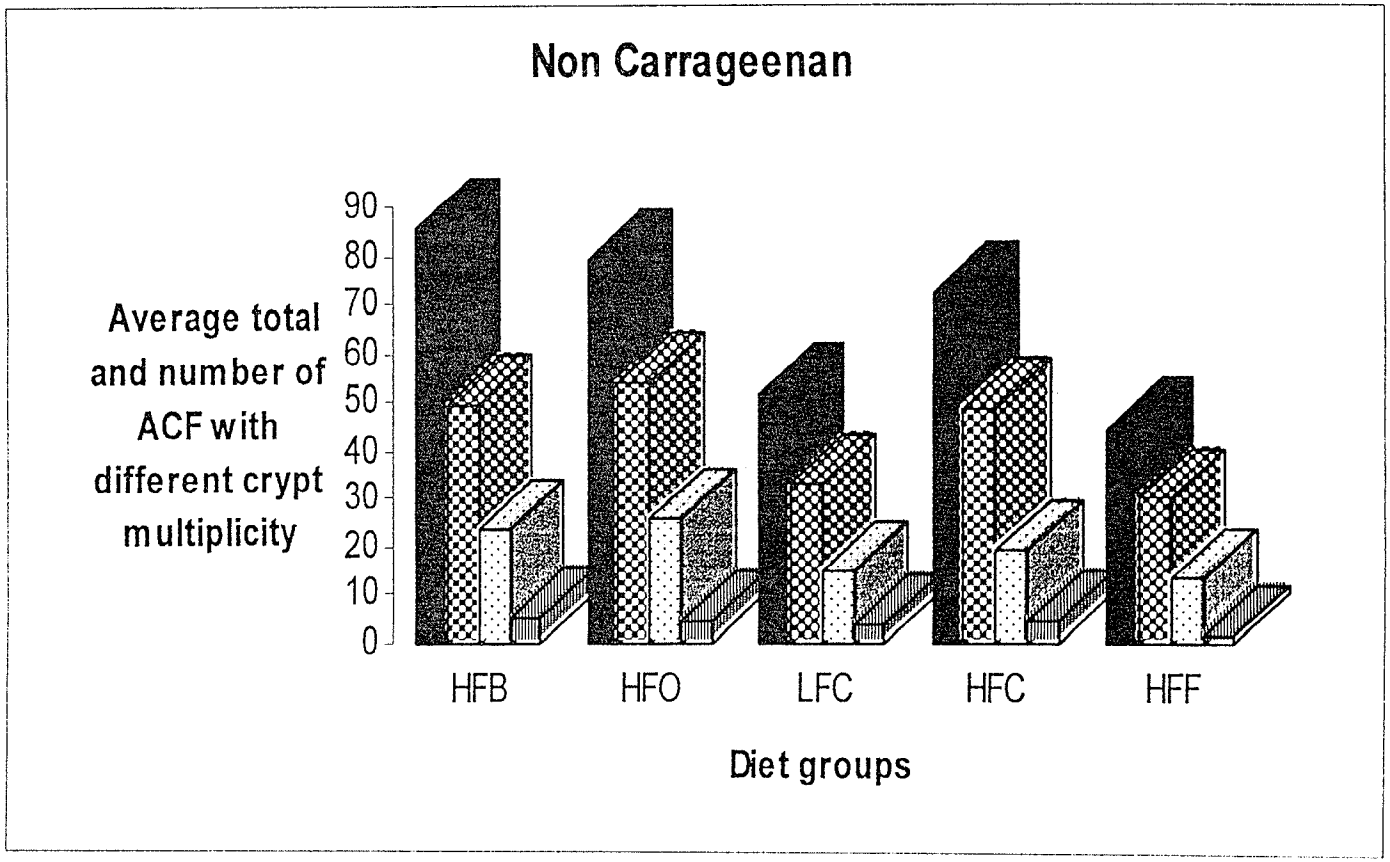

Non Carrageenan
Average total and number of ACF with different crypt multiplicity
90
80
70
60
50
40
30
20
10
0
HFB
HFO
LFC
HFC
HFF
Diet groups

Figure 6.5. Total number of ACF in female Sprague-Dawley rats fed one of five diets: LFC+, HFC+, HFB+, HFO+ or HFF+, 2 weeks prior and 4 weeks after AOM injection. Values are means ± SE. Bars labeled with different letters are significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).

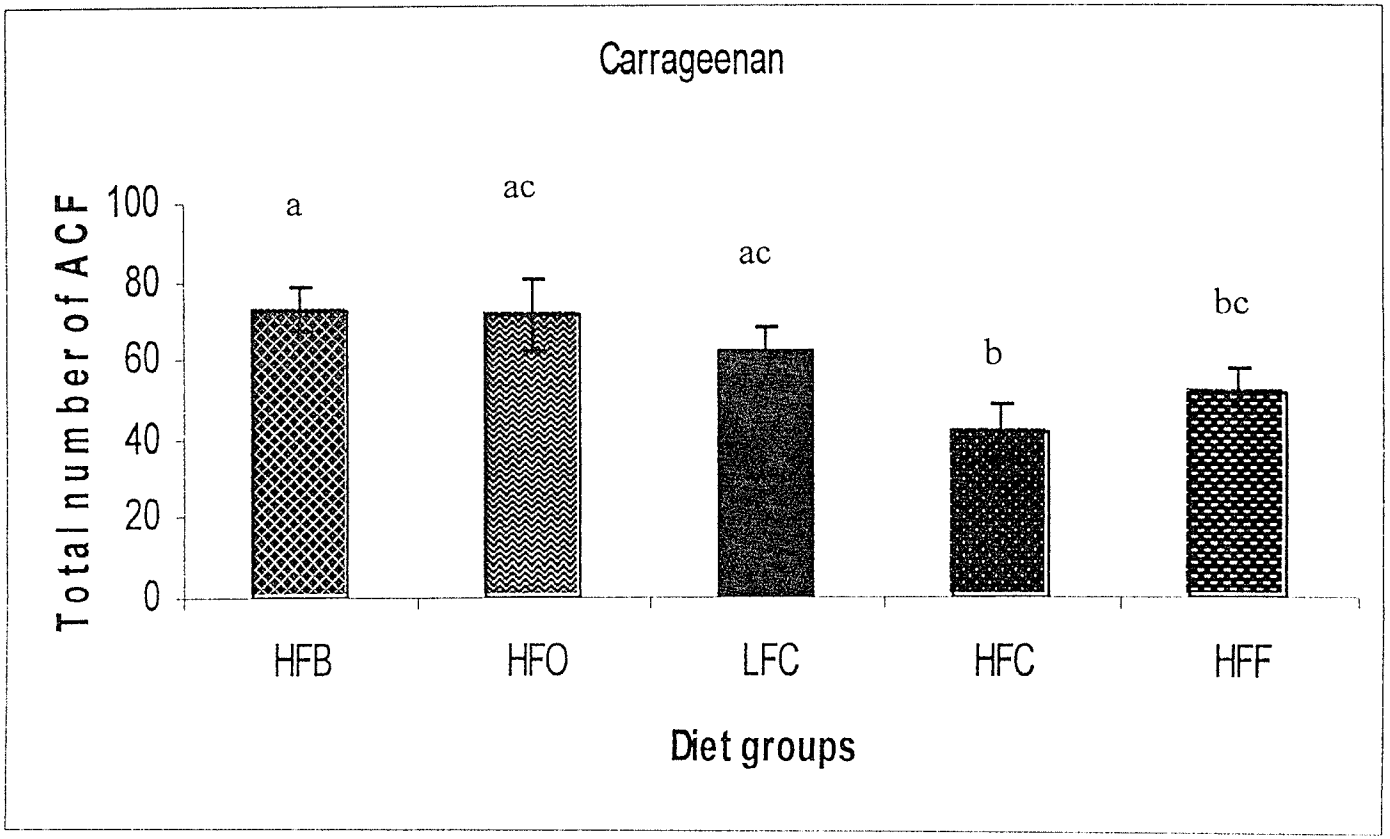

Carrageenan
Total number of ACF
100
80
60
40
20
0
a
ac
ac
b
bc
HFB
HFO
LFC
HFC
HFF
Diet groups

Table 6.5. The number and growth features of ACF in Sprague-Dawely rats as affected by the type of dietary Lipids, with or without carrageenan, fed two weeks prior and four weeks after AOM injection.

Diet Group	TACF	ACF1	ACF2	ACF3+
HFB	85.63 ± 9.70	49.38 ± 5.02	23.63 ± 3.54	5.00 ± 1.21
HFB+	72.71 ± 5.45	42.43 ± 8.14	27.88 ± 3.33	6.75 ± 2.00
HFO	79.14 ± 6.47	53.86 ± 5.38	25.75 ± 4.88	4.63 ± 1.64
HFO+	71.63 ± 9.49	45.25 ± 4.98	21.88 ± 3.71	4.50 ± 1.15
LFC	51.40 ± 9.72	33.00 ± 7.90	15.00 ± 1.92	3.40 ± 0.60
LFC+	62.67 ± 5.30	40.50 ± 4.43	18.67 ± 2.39	3.50 ± 1.12
HFC	72.38 ± 8.01	48.75 ± 5.49	19.38 ± 2.92	4.25 ± 0.82
HFC+	42.13 ± 7.01	28.38 ± 3.62	11.25 ± 2.91	3.13 ± 1.33
HFF	44.63 ± 7.73	30.13 ± 4.28	13.13 ± 8.58	1.38 ± 0.84
HFF+	52.50 ± 5.24	38.63 ± 3.20	12.75 ± 2.07	1.13 ± 0.61

1. Values are means ± SE.
2. Abbreviations are as follows: TACF (total aberrant crypt foci); ACF1 and ACF2 (aberrant crypt foci with one or two apparent aberrant crypts); HFB (high fat beef tallow); HFO (high fat olive oil); LFC (low fat corn oil); HFC (high fat corn oil); HFF(high fat flaxseed oil).
3. Diet groups with a sign (+) contained 2% of carrageenan by weight.

Figure 6.6. COX-2 expression in colonic mucosa as modulated by carrageenan and the amount of ω-6 fatty acid fed 2 weeks prior and 4 weeks after AOM injection. Values are means ± SE. Bars labeled with the same letter are not significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).

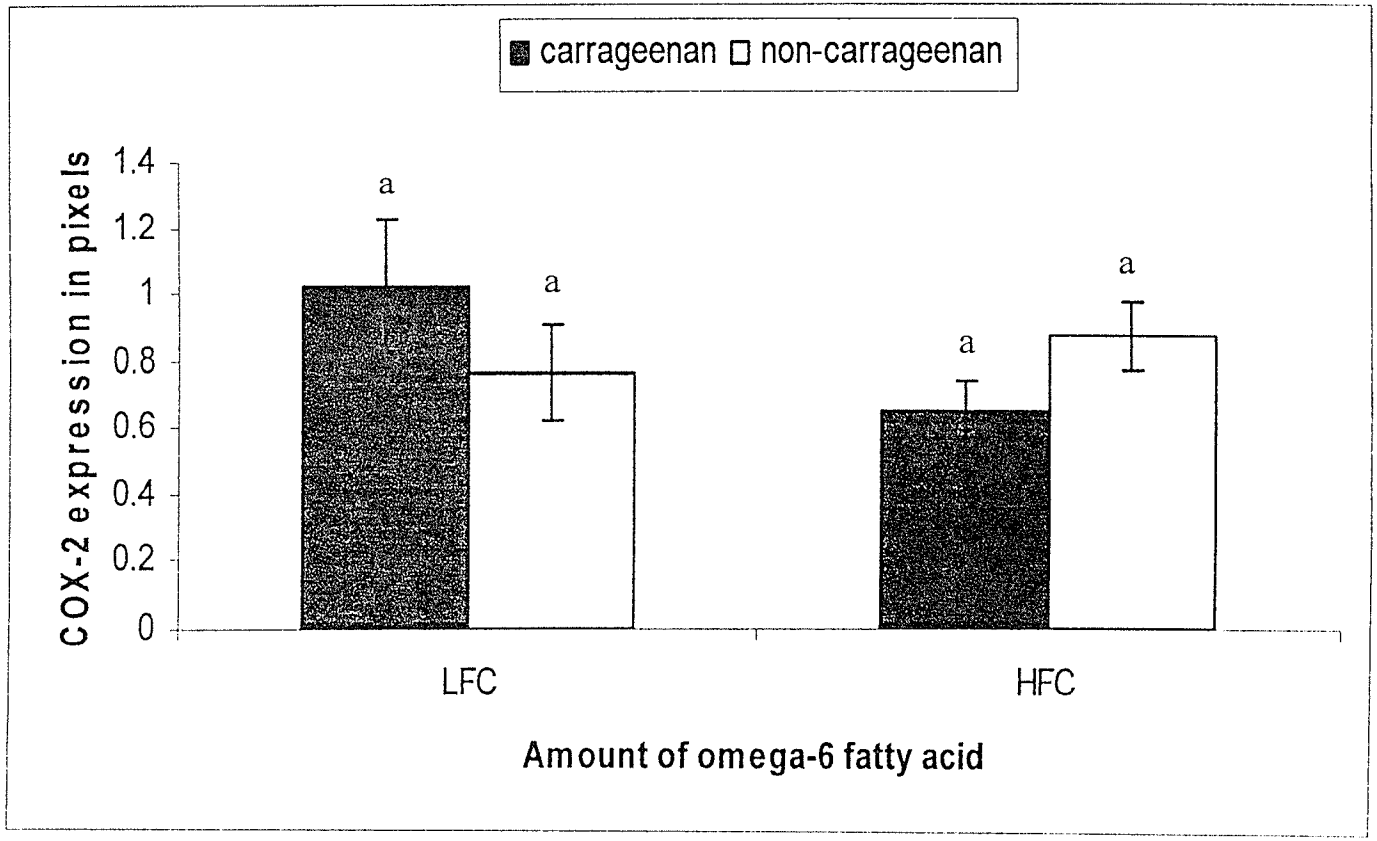
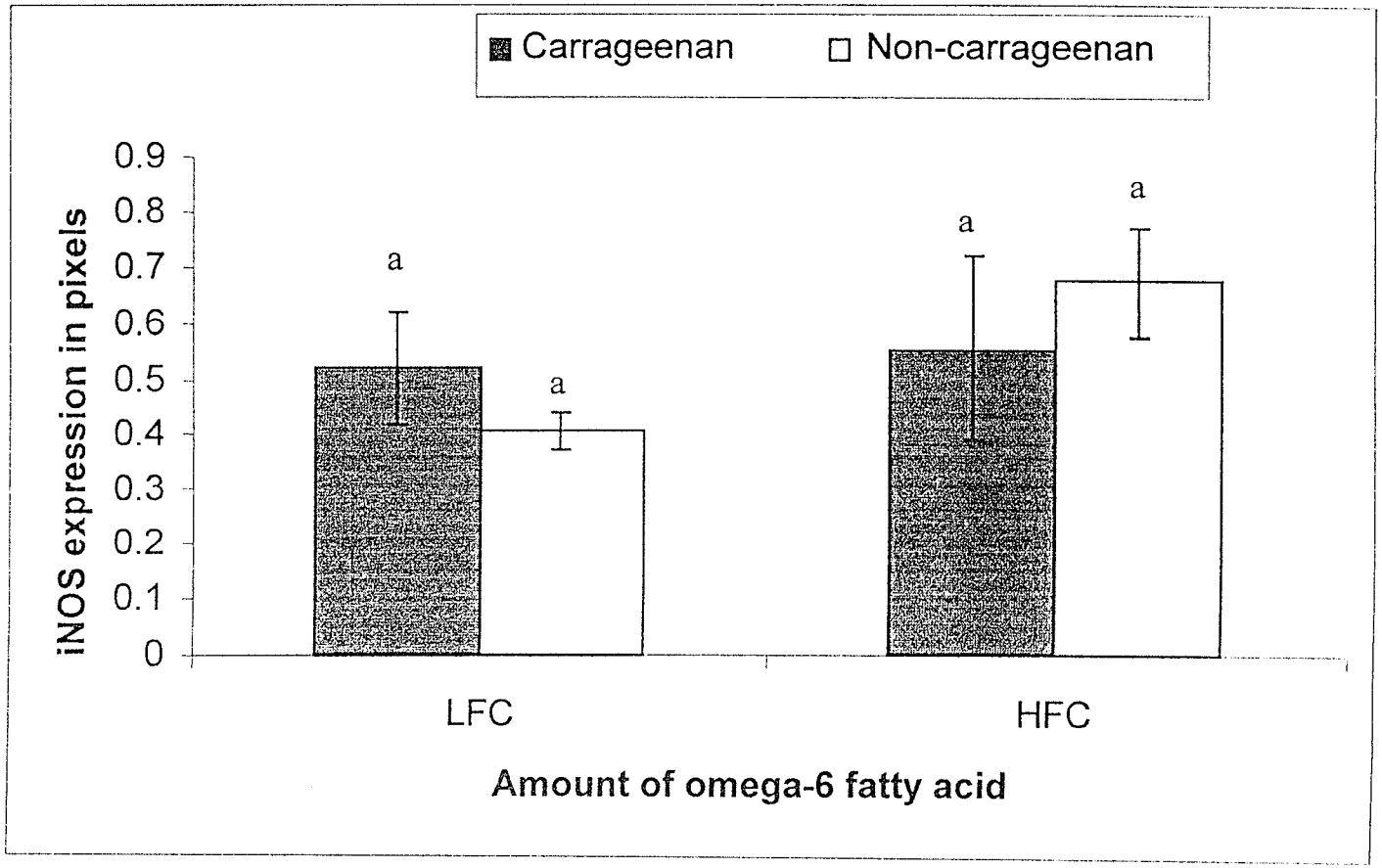

carrageenan
non-carrageenan
COX-2 expression in pixels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
a
LFC
HFC
Amount of omega-6 fatty acid

Figure 6.7. iNOS expression in colonic mucosa as modulated by carrageenan and the amount of ω-6 fatty acid fed 2 weeks prior and 4 weeks after AOM injection. Values are means ± SE. Bars labeled with the same letter are not significantly different at $P \leq 0.05$ (ANOVA and Tukey's test).


Carrageenan
Non-carrageenan
iNOS expression in pixels
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
a
a
LFC
HFC
Amount of omega-6 fatty acid

6.3. Discussion

In general, modulating the effects of high levels of dietary lipids on chemically induced colon carcinogenesis has shown that diets high in saturated fat (beef tallow), corn oil or safflower oil are tumor enhancing, while diets high in fish oil, coconut oil, or olive oil had no colon tumor promoting effects compared to their respective low fat diets (Reddy, 1992). The main premise of the present study was our preliminary finding that the HFC and HFF diets with CAR reduced the number and growth of chemically (AOM) induced ACF. We were unsure if ACF reduction is specific to these two high fat diets or were common to all high fat diets regardless of their fatty acid composition. Using the method of Dahleh (2001) animals were fed designated diets two weeks before and four weeks after the injection of a chemical carcinogen so that the effect of dietary lipids was expected to affect initiation and promotional stages of inflammation-associated colon carcinogenesis. Findings confirmed the results of the preliminary study of that feeding LFC+ increased the sensitivity of the colon toward the carcinogenic changes resulting in an overall higher number of ACF with 1 or 2 crypts multiplicity. This effect, however, was not as pronounced as in male rats noted previously by Dahleh (2001). This could be attributed to the fact that females are less sensitive to AOM induced colon carcinogenesis and CAR feeding. HBT, HFO and HFC diets had the most promoting effects on the number as well as on the growth features of the ACF. However, these high fat diet groups with CAR had decreased 1 and 2 crypts multiplicity, which was most pronounced for HFC+ diet, which significantly decreased the total number of ACF compared to its non-CAR counterpart (Figure E.1.; Appendix E). The HFF diet showed protective effects with or without addition of carrageenan, noticeable in the promotional stage of carcinogenesis

as seen in significant decline of advanced lesions (3 or more crypts/focus) but not in 1 and 2 crypts multiplicity. Based on the number of advanced lesions the HFF diet would be predicted to have a lowest tumor outcome (Figure E.2.; Appendix E).

The most important finding of this study is the consistent protective effect of HFF diet which contained 14.3% corn oil (ω-6) diet and 9.5% flaxseed oil (ω-3), indicating the potent retarding effects of even small amounts of ω-3 fatty acids on sporadic as well as on colitis-associated colon cancer development. Moreover, confirming our preliminary work, present study showed that α-linolenic 18:3 ω-3 fatty acid found in flaxseed had similar beneficial effects as its longer chain relatives (EPA and DHA) found in fish oil (for review see 3.1.0.).

It is worth noting that only a limited number of studies have reported a cancer-preventive effect of HFO in AOM induced colon carcinogenesis (Reddy, 1992). In the present study, however, it was noted that HFO diet promoted the growth of ACF. This finding was unexpected, and could be attributed to effect of HFO on the later stages of tumorigenesis affecting advanced lesions only.

High fat diets are reported to be immunosuppressive, however, whether a high fat diet reduces the inflammatory responses in the colonic mucosa requires further investigation. Regardless of the fatty acid composition of the diet, the ACF enhancing effect of high fat diets, such as HFB and HFO, did not change within 4 weeks of CAR feeding. A long-term study is desirable in the future to determine if CAR feeding affects inflammatory responses in the colon and whether it influences selection and propagation of preneoplastic lesions to more advanced stages in these groups.

Biochemical analysis of AOM treated colonic mucosa from animals fed LFC, LFC+, HFC or HFC+ diet, revealed that the levels of corn oil and CAR affected the expression of COX-2 and iNOS. The HFC diet increased the COX-2 compared to LFC; moreover, CAR feeding had an opposite effect in LFC group compared to HFC group. The COX-2 level increased in LFC+ and decreased in HFC+ groups compared to LFC and HFC groups respectively. These findings suggest that increased expression of COX-2, post-initiation, could be important in favouring the growth of ACF. A similar trend was noted for iNOS.

A previous study conducted by Good (1999), demonstrated that HFC feeding for 12 weeks augmented ($P \leq 0.05$) the growth of ACF and tumors as well as the expression levels of COX-2 in normal appearing mucosa compared to LFC diet. In the present study we saw a moderate increase or decrease in COX-2 expression due to dietary treatments that could be due to the shorter feeding period (4-6 weeks).

The trend observed in the present study suggests that iNOS and COX-2 could have a role in colon cancer development. This role could be quite different in sporadic versus colitis-associated carcinogenesis pointing complex interactions of these molecules and their roles dependent on the pathological process.

6.4. Summary

The findings of the preliminary study were confirmed that HFC+ diet reduces the appearance of ACF, and that HFF diet with or without CAR is the least permissive to ACF growth compared to all high fat diets. The LFC+ diet was also shown to increase the

number of ACF compared to LFC diet but this effect was not as pronounced as previously noted. The new finding in the present study was the enhancing effect of CAR on ACF development, which was not evident among all high fat diets. HFC and HFF effects noted in the previous and the present study are unique to these diets. These diets exerted different effects from each other, which may reflect their unique fatty acid composition.

Analysis of colonic mucose from LFC, LFC+, HFC and HFC+ suggest that increased levels of COX-2 and iNOS during post-initiation stages favour the appearance and growth of preneoplastic lesions.

CHAPTER 7

General discussion and conclusion

Ulcerative colitis is known to increase the risk for developing colon cancer. Colitis like symptoms could be induced in the colon of several laboratory animals by chemicals generally leading to acute ulceration of a shorter duration. In addition, attempts have been made to use animals that are genetically altered in a manner that they develop colitis like symptoms for a specific duration. An ongoing interest in our laboratory has been to identify an experimental approach that would induce chronic colitis like changes in the colon without intestinal bleeding or severe weight loss, common to individuals with inactive UC. In a preliminary study, feeding undegraded carrageenan (2% by weight) to male rats caused a number of changes pertinent to chronic inflammation of the colon. However, it remained unclear as to when visible morphological changes occur and if morphological changes precede biochemical changes. In the same preliminary study, the risk of AOM-induced colon cancer was higher in the CAR-treated LFC+ group by comparison to the LFC, non-CAR treated group. This risk, however, decreased in the HFC+ group, indicating a protective effect by high fat diet. Moreover, the effect of dietary lipids rich in ω-3 fatty acids ameliorated inflammation and formation of inflammation-associated increase of colonic preneoplastic lesions. The carrageenan-induced animal model provides the opportunity to explore the underlying mechanism(s) by which inflammation may influence the risk of developing colon cancer as well as to explore potential therapeutic and preventive approaches that could be applicable to human population.

The objective of this research was to define and refine the carrageenan model of ulcerative colitis in Sprague Dawley rats, and to assess systematically the risk of colon cancer in this model. The overall working hypothesis was that inflamed colonic mucosa will be more sensitive to colon carcinogenesis when colon is exposed to CAR for a longer time period and that a high fat diet will modulate the ability of CAR to augment colon carcinogenesis independent on its fatty acid composition. The specific objectives of the research were:

1. To sequentially analyze the inflammatory effect of carrageenan in rat colonic mucosae to determine the duration required to produce morphological and biochemical changes.
2. To assess the effect of the duration of CAR feeding before, during and after the administration of carcinogen injection on the sensitivity of colonic mucosa to AOM induced colon carcinogenesis.
3. To understand the effect of high fat diets, varying in fatty acid composition, on the ability of CAR to augment the formation of colonic preneoplastic lesions.

The main findings were that CAR affected the morphology marked by the induction of lymphocytic infiltration in colonic mucosa within two weeks of feeding. Major biochemical alterations took place by six weeks; a decrease in the COX-2 and increases in iNOS and TNF-alpha protein expression were noted in CAR group by comparison to the control. The maximum duration of CAR feeding used in the present study, was six weeks prior to AOM injection. This significantly increased the sensitivity to colon carcinogenesis, despite animals being switched to the control diet immediately after the AOM injection. Initiation of CAR feeding concurrently with AOM injection

accelerated the number and growth of ACF. This effect was noted in rats fed CAR for two weeks and then switched to a control diet. These findings attest to the importance of inflammatory stimulus in heightening the risk of colon cancer and demonstrate that occurrence of inflammation along with genotoxic chemical responses appear to be critical for increasing the risk.

It became apparent that CAR did not increase the number of ACF in the high fat diet groups compared to in low fat diet. One suggestion is that high fat diets are immunosuppresive and may have affected the biological activity of CAR and immune cells. It was also clear that, with the exception of HFF, all high fat diets enhanced the appearance of ACF with advanced growth features compared to LFC diet. A decrease in the total number of ACF in the HFC+ group compared to HFC group and an increase in the total number of ACF in the LFC+ group compared to the LFC group was observed. This study attempted to assess whether the colonic mucosa from these groups would exhibit changes at the biochemical level, which would distinguish them for their ability to support the growth of ACF. While the level of TNF-alpha protein could not be detected changes in the levels of COX-2 and iNOS were observed. The HFC+, group with fewer ACF, also had lower level of COX-2 in the colonic mucosa than the HFC and LFC+ groups, as well as lower level of iNOS than the HFC group. The increase or decrease in the levels of these two molecules accompanied by an increase or decrease in the number of ACF suggested that these molecules may be important in colon carcinogenesis, is supported by other research (see 2.1.2.0.), and that HFC+ mucosa was unable to up-regulate COX-2 and iNOS. Examining the effect of CAR on non-carcinogen treated mucosa it was clear that this treatment in the LFC group down regulated COX-2 and up-

regulated iNOS and TNF-alpha. However, in the carcinogen treated colonic mucosa, CAR feeding up-regulated COX-2 in the LFC group and lowered the level of COX-2 in the HFC group. These findings suggest that the effect of CAR feeding was different in AOM treated colonic mucosa compared to non-AOM treated mucosa. It can also be speculated that COX-2 may have a different effect in altering the sensitivity of colonic mucosae to carcinogen as opposed to favoring the growth of preneoplastic lesions during the post initiation stages. It was interesting to note that iNOS was elevated in the LFC+ group during pre-initiation and post-initiation stages, suggesting that iNOS may be a good candidate to consider as a growth enhancing molecule for ACF.

The most salient findings of the present research are:

A. Moderate inflammation without severe ulceration is sufficient for enhancing the risk of colon cancer;
B. The effect of CAR is not evident in high fat diets;
C. Substitution of corn oil (ω-6) with flax oil (ω-3), in HFF diet, abrogated the ACF enhancing effect of corn oil diet;
D. The CAR model has potential to be exploited as a model to mimic a moderate chronic colitis state in future studies.

The present study provides important clues regarding the CAR model and its efficacy in studying the link between UC and colon carcinogenesis. However, the biological effect of CAR, and the molecular interaction that may take place among molecules generated during inflammation and following AOM injection is not clear. How

these molecules may be shared by the tissue in counteracting inflammatory responses and in modifying the growth of ACF should be explored further in future studies.

In addition, our findings demonstrated that amount of fat with specific fatty acids composition might play an important role in colitis-associated carcinogenesis. The effect of HFF diet in reducing the risk of colitis-associated preneoplastic lesions is most noteworthy and needs to be further explored. Moreover, the ACF enhancing effect of HFO diet needs further attention.

CHAPTER 8

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CHAPTER 9

Appendix A

Table A.1.The composition of experimental diets

Ingredients	LFC	HFC	HFB	HFO	HFF
Casein	195	238.8	238.8	238.8	238.8
L-cystine	2.9	3.6	3.6	3.6	3.6
Corn Starch	431.7	125.4	125.4	125.4	125.4
Maltodextrin	128.8	157.6	157.6	157.6	157.6
Sucrose	97.6	119.4	119.4	119.4	119.4
Cellulose	48.8	59.7	59.7	59.7	59.7
AIN 93G Mineral Mix	34.2	41.8	41.8	41.8	41.8
AIN 93G Vitamin Mix	9.8	11.9	11.9	11.9	11.9
Choline Bitartrate	2.4	3.0	3.0	3.0	3.0
Corn Oil	48.8	238.8	48.8	48.8	143.3
Test Oil	/	/	190	190	95.5

1. Values of all ingredients are expressed in grams and are calculated per 1 kg of diet.
2. All diets are based on AIN93G semi-purified diet modified for fat content and adjusted in amount of other ingredients to ensure that all animals were fed isocaloric diet.
3. Abbreviations are as follows: LFC (low fat corn oil); HFC (high fat corn oil); HFF (high fat flaxseed oil); HFO (high fat olive oil); HFB (high fat beef tallow diet); AIN93G (American Institute of Nutrition-93 growth diet).

Appendix B

PBS was used for cleaning (flashing) colons at termination, for ACF scoring as described in Chapter 4 as well as for washing of slides in immunohistochemistry.

All chemicals were purchased from Sigma Chemical Co.

Phosphate buffered saline - PBS (pH 7.3)

137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4 x 7 H_2O
1.4 mM KH_2PO_4

Appendix C

Buffers used for homogenization of colonic mucosal scrapings.

All chemicals were purchased from Sigma Chemical Co.

Homogenizing buffer (stock 25 ml)

Ripa buffer	25 ml
Aprotinin	31.25µl
Leupeptin	125µl
Trypsin Inhibitor	6.25µl
SOV (sodium orthovanadate)	250µl
NaF	250µl

RIPA buffer

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

Appendix D

Western blots buffers and gel recipes (based on the method described by Laemmli, 1970).

All chemicals used were purchased from Bio-Rad Laboratories Ltd, Canada.

SDS-PAGE Stacking gel	5%
dd H_2O	3.4 ml
30% Acrylamide/bis (Cat # 161-0156)	0.83 ml
0.5 M Tris (pH 6.8)	0.63 ml
10% SDS (sodium dodecyl sulphate)	0.05 ml
10% Ammonium persulfate (APS) (Cat # 161-0700)	0.05 ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	0.005 ml

SDS-PAGE Resolving (separating) gel	10%
dd H_2O	3.3 ml
30% Acrylamide/bis	4.0 ml
1.5 M Tris (pH 6.8)	2.5 ml
10% SDS	0.1 ml
10% Ammonium persulfate (APS)	0.1 ml
TEMED	0.004 ml

Sample buffer

2% SDS
10% Glycerol (w/v)
5% β-Mercaptoethanol
0.025% Bromophenol blue
62.5 mM Tris (pH 6.8)

5X Electrode (Running) buffer (1L)

Tris-buffer (TBS)	15 g
Glycine	77 g
SDS	5 g

Make final volume to 1000ml with addition of dd H2O.

Tris-buffered saline (TBS)

100 mM Tris HCl (pH 7.5)
0.9% NaCl

Transfer buffer (1L)

Tris-buffer (TBS)	3.03 g
Glycine	14.4 g
Methanol	200 ml

Make final volume to 1000ml with addition of dd H2O.

Blocking solution

5% skim milk powder in TBS-T

TBS-T buffer (pH 7.5)

100 mM Tris HCl
0.9% NaCl
0.1% Tween 20

Coomassie blue stain

0.05% Coomassie brilliant blue R-250
50% methanol
10% acetic acid
40% dd H2O

Destain solution

5% methanol
7% acetic acid
88% dd H2O

Appendix E

Figure E.1. The total number of ACF in female Sprague-Dawley rats as affected by the type or amount of dietary lipids with or without carrageenan.

Values are means ± SE. Different letters indicate significantly different values ($P \leq 0.05$; ANOVA and Tukey's test) among non-carrageenan and carrageenan treatment within the same diet group.

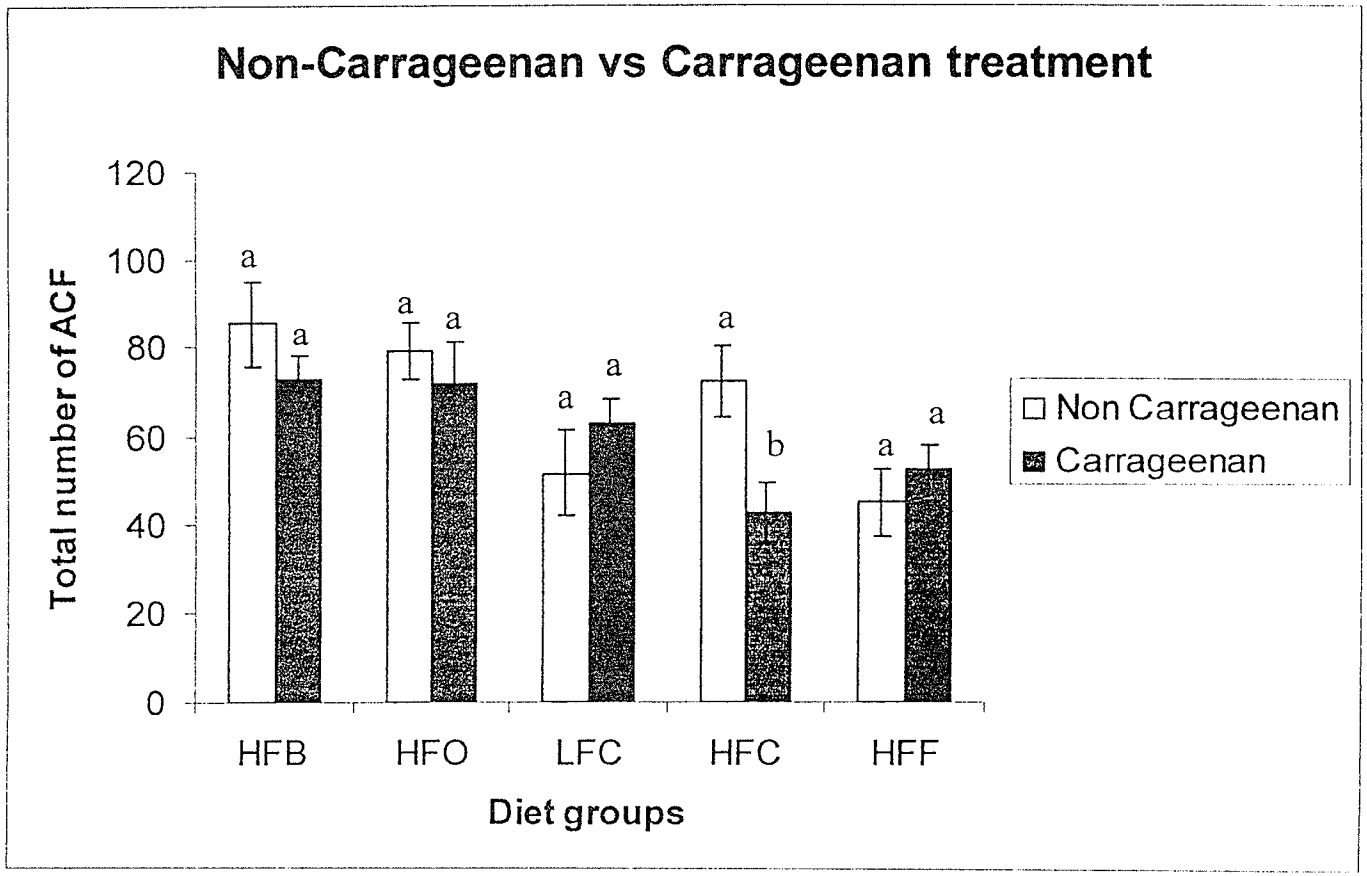

Non-Carrageenan vs Carrageenan treatment
Total number of ACF
120
100
80
60
40
20
0
a
a
a
a
a
a
a
b
a
a
HFB
HFO
LFC
HFC
HFF
Diet groups
Non Carrageenan
Carrageenan

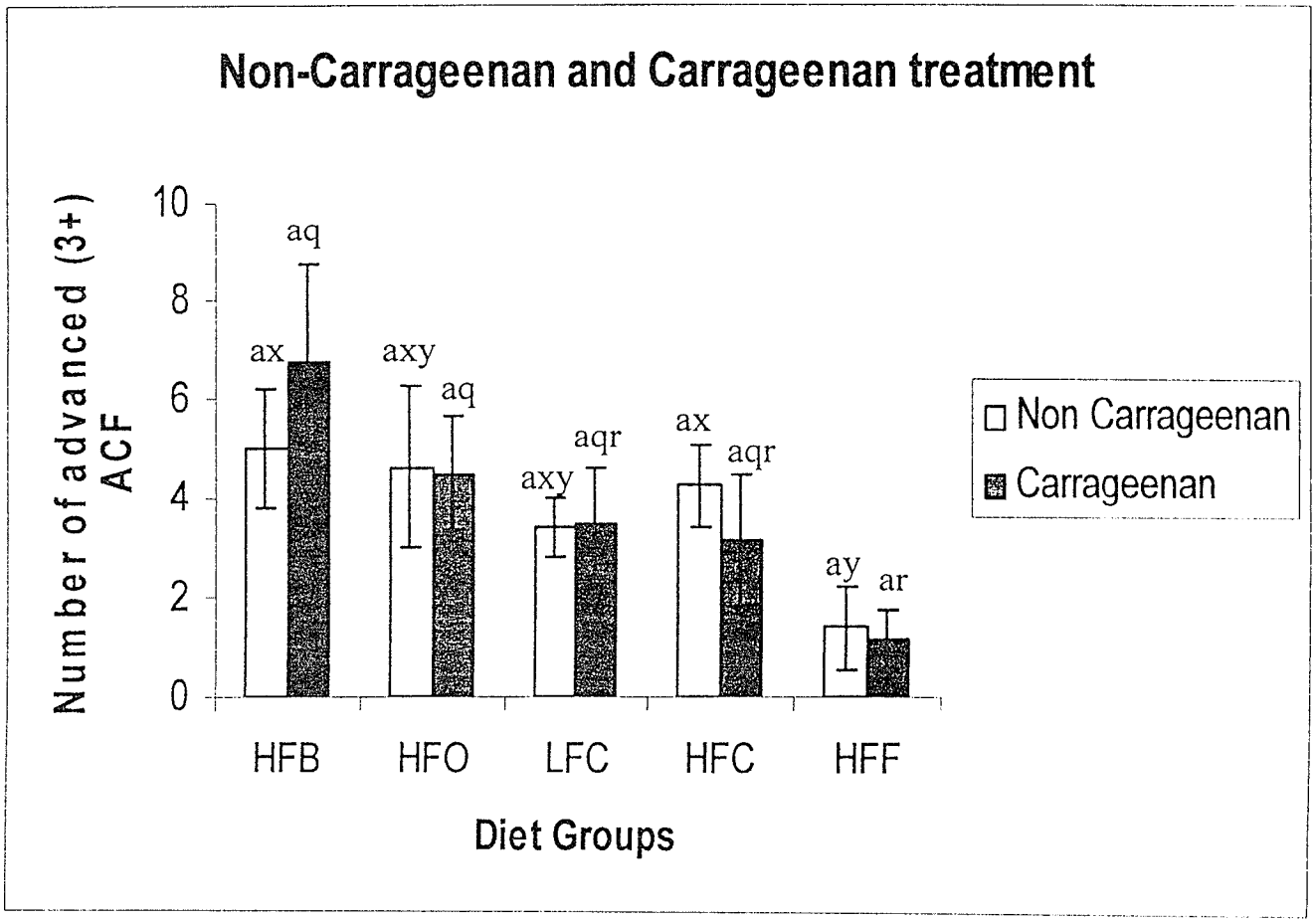
Figure E.2. Number of advanced (3+) ACF in female Sprague-Dawley rats as affected by the type or amount of dietary lipids with or without carrageenan.

Values are means ± SE. Different letters indicate significantly different values at $P \leq 0.05$ (ANOVA and Tukey's test).

a,b indicate significantly different values among non-carrageenan and carrageenan treatment within the same diet group.

x,y indicate significantly different values among various diet groups non-carrageenan treated.

q,r indicate significantly different values among various diet groups carrageenan treated.


Non-Carrageenan and Carrageenan treatment
Number of advanced (3+) ACF
10
8
6
4
2
0
aq
ax
axy
aq
aqr
axy
ax
aqr
ay
ar
HFB
HFO
LFC
HFC
HFF
Diet Groups
Non Carrageenan
Carrageenan