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Effects of Dietary Fatty Acids on Intestinal Tumorigenesis in $Apc^{Min/+}$ Mice

Melissa Hansen Petrik
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Melissa Hansen Petrik entitled "Effects of Dietary Fatty Acids on Intestinal Tumorigenesis in *Apc*$^{Min/+}$ Mice." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Jay Whelan, Major Professor

We have read this dissertation and recommend its acceptance:

Betsy Haughton, Michael F. McEntee, Naima Moustaid Moussa

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Effects of Dietary Fatty Acids on Intestinal Tumorigenesis in *Apc*$^{Min/+}$ Mice

A Dissertation
Presented for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Melissa Hansen Petrik
May 2001

DEDICATION

This dissertation is dedicated to

my husband

Jeff Petrik

my parents

Richard A. and Janette K. Hansen

and my sister

Kristina A. Hansen

for their never-ending support and encouragement.

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ABSTRACT

Dietary fat is thought to be an important environmental factor influencing colorectal cancer risk. n-3 polyunsaturated fatty acids (PUFA) have been linked to a lower risk of colorectal cancer and it has been presumed that this effect is due to their ability to interfere with synthesis of 2-series prostaglandins from arachidonic acid (AA, 20:4 n-6). However, the specific mechanism has not been clearly established. Therefore, we hypothesized that select dietary fatty acids would modulate intestinal tumorigenesis through interference with AA metabolism in the *Apc*$^{Min/+}$ mouse, a unique model system bearing many similarities to human intestinal cancer. α-linolenic acid (ALA, 18:3 n-3), stearidonic acid (SDA, 18:4 n-3), eicosapentaenoic acid (EPA, 20:5 n-3), and docosahexaenoic acid (DHA, 22:6 n-3) significantly reduced phospholipid AA concentrations and prostaglandin levels compared to controls, but only SDA and EPA significantly reduced overall tumor number and size. Two other PUFA also proposed to be anti-tumorigenic, conjugated linoleic acid (cLA, 18:2 n-6 and n-7) and γ-linolenic acid (GLA, 18:3 n-6), did not significantly alter AA concentrations, prostaglandins, or tumorigenesis. The effects of EPA on phospholipid AA levels, prostaglandins, and tumorigenesis were eliminated when equivalent amounts of AA were provided concomitantly in the diet, indicating that EPA exerts its anti-tumorigenic effect, at least in part, through antagonism of arachidonic acid and/or its metabolism. Selective inhibition of Δ-6 desaturase, the rate-limiting step in *de novo* AA biosynthesis, likewise resulted in 37% fewer tumors compared to controls and this was normalized when AA was provided concomitantly in the diet, indicating that inhibition of *de novo* AA biosynthesis inhibits tumorigenesis in this model. Finally, the nonsteroidal anti-inflammatory drugs (NSAIDs) piroxicam and sulindac, which are also inhibitors of prostaglandin biosynthesis, are able

to regress preexisting intestinal tumors. Bypassing prostaglandin inhibition with concomitant NSAID and PGE_2 receptor agonist treatment muted the anti-tumorigenic effects of both NSAIDs, indicating that prostaglandin inhibition is responsible, in part, for the anti-tumorigenic effect of NSAIDs. Additionally, treating *Apc*$^{Min/+}$ mice with an anti-PGE_2 antibody, which prevents PGE_2 from binding to its receptors and eliciting cellular responses, also resulted in 33% fewer tumors. This research firmly establishes a role for AA and for AA-derived prostaglandins in promotion of intestinal tumorigenesis.

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AA	Arachidonic acid
AAPC	Attenuated adenomatous polyposis coli
ALA	α-linolenic acid
AOM	Azoxymethane
Apc	Murine adenomatous polyposis coli
APC	Human adenomatous polyposis coli
$Apc^{\Delta 716}$	*Apc* knockout mouse with *Apc* truncation at codon 716
$Apc^{Min/+}$	Multiple intestinal neoplasia mouse
cLA	Conjugated linoleic acid
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
DAG	Diacylglycerol
DCC	Deleted in colorectal carcinoma
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DLG	*Drosophila* disc-large protein
DMBA	7,12-dimethylbenz(α)anthracene
DPA	Docosapentaenoic acid
EGF	Epidermal growth factor
ENU	*N*-ethyl-*N*-nitrosurea
EPA	Eicosapentaenoic acid
EP receptor	E prostaglandin receptor
EP*R*-A	E prostaglandin receptor agonist

FAP	Familial adenomatous polyposis
GLA	γ-linolenic acid
GSK-3β	Glycogen synthase kinase-3β
H&E	Hematoxylin and eosin
15-HETrE	15-hydroxyeicosatrienoic acid
HNPCC	Hereditary nonpolyposis colon cancer
iNOS	Inducible nitric oxide synthase
IP_3	Inositol triphosphate
LA	Linoleic acid
6-keto-$PGF_{1\alpha}$	6-keto-prostaglandin $F_{1\alpha}$
MCC	Mutated in colorectal cancer
MDR1	Multidrug resistance gene
Mom1	Modifier of Min 1
NF-κB	Nuclear factor κB
NSAID	Nonsteroidal anti-inflammatory drug
OA	Oleic acid
PBS	Phosphate buffered saline
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
PKC	Protein kinase C
PPAR	Peroxisome Proliferator Activated Receptor

PUFA	Polyunsaturated fatty acids
SAMP	Ser-ala-met-pro repeats
13-*S*-HODE	13-*S*-hydroxyoctadecadienoic acid
SDA	Stearidonic acid
TXA_2	Thromboxane A_2

PART I

Introduction

Colorectal cancer is the second leading cause of cancer deaths in the United States, and dietary fat, both the type and amount consumed, is among several environmental factors implicated in influencing colorectal cancer incidence. The n-3 family of polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) present in fish and fish oil, and some n-6 fatty acids including conjugated linoleic acid (cLA, 18:2 n-6 and n-7) and γ-linolenic acid (GLA, 18:3 n-6), have been investigated for potential anti-tumorigenic effects in recent years. These fatty acids are proposed to be anti-tumorigenic due to interference with arachidonic acid (AA, 20:4 n-6) or its metabolism, and more specifically due to interference with formation of downstream metabolic products called prostaglandins, such as PGE_2 which is derived from AA.

A role for prostaglandins as cancer promoters is supported by several studies demonstrating that non-steroidal anti-inflammatory drugs (NSAIDs) such as sulindac and piroxicam have chemotherapeutic effects in various models of colorectal cancer, presumably due to their ability to inhibit cyclooxygenase (COX), the committed step in prostaglandin formation. Although various animal models are used to study the effects of nutritional intervention on intestinal tumorigenesis, the $Apc^{Min/+}$ mouse model is particularly valuable because of its germline mutation in the murine *Apc* gene. Humans with familial adenomatous polyposis (FAP) also possess a germline mutation in *APC* and mutational damage or loss of the wild type allele initiates intestinal tumor formation. However, somatic mutations resulting in loss of full length *APC* protein also occur early in spontaneous forms of the disease indicating an *APC* defect is associated with a majority of human colorectal cancers. APC acts as a tumor suppressor by facilitating the phosphorylation and subsequent degradation of β-catenin which, in the absence of

functional APC, upregulates transcription of several target genes that may be involved in tumorigenesis.

The overall goal of my research was, therefore, to determine the anti-tumorigenic efficacy of various dietary fatty acids in the *Apc*$^{Min/+}$ mouse and determine whether interference with arachidonic acid could explain, at least in part, the observed effects.

PART II

Review of the Literature

INTESTINAL CANCER

Cancer is the second-leading cause of death in the United States surpassed only by heart disease. Colorectal cancer in the second-leading cause of cancer deaths in both men and women trailing only lung cancer and it accounts for 56,000 deaths annually (1). The development of colorectal cancer is thought to be influenced by both genetic predisposition and environmental factors, including dietary habits and physical activity. Although most colon cancers occur due to somatic mutations, there are two inherited disorders that are known to be responsible for a small percentage of total colon cancers: familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC).

MOLECULAR GENETICS OF COLON CANCER

Several genetic risk factors have been identified for development of colon cancer including inactivated tumor suppressor genes, activated oncogenes, and mutated DNA mismatch repair genes that direct the initiation, promotion, and progression of colon cancer. Identification of these genes led in 1990 to development of a model in which an orderly progression of accumulating mutations in specific genes results in tumor formation (**Fig. 2-1**) (2). This model has continued to evolve throughout the past decade as new information on gene functions and their interactions comes to light (3).

Oncogenes, tumor suppressor genes and colon cancer. The normal function of proto-oncogenes is to control mitogenesis and differentiation. However, alterations to these genes due to amplification, point mutation, or translocation results in dysregulation of cellular proliferation and tumorigenesis. Once mutated, they are called oncogenes.

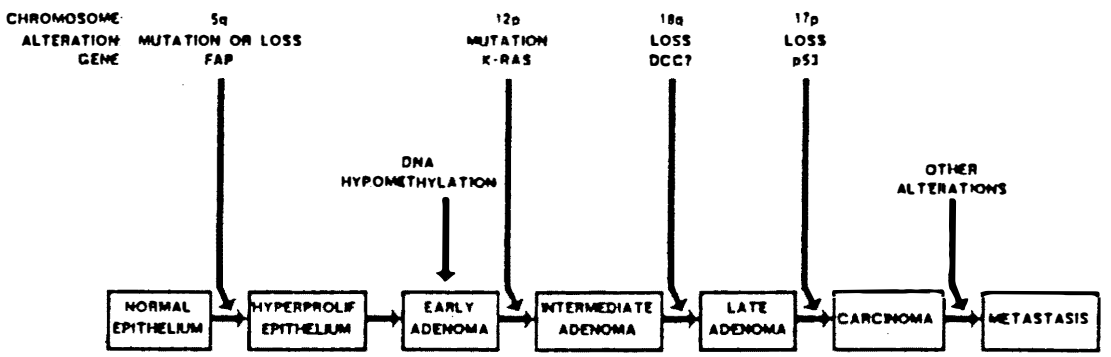

FIGURE 2-1. Model of molecular mutations involved in the progression of colon cancer as described by Fearon and Vogelstein (2).

Oncogenes are dominant, so only one allele need be altered in order to induce tumorigenesis. The normal function of tumor suppressor genes is to prevent uncontrolled cellular growth, thus, in contrast to oncogenes, tumorigenesis occurs following loss of gene function. Since tumor suppressor genes are autosomal recessive, both alleles of the gene must be lost or mutated in order for tumor formation to commence.

In addition to alterations in cellular proliferation rates, the failure of damaged cells to undergo programmed cell death, or apoptosis, has also been implicated in colon tumorigenesis (4). Apoptosis is a gene-directed program for elimination of unwanted or damaged cells and several genes have been identified as either inducers or inhibitors of apoptosis. Mutations of these oncogenes and tumor suppressor genes prolong cell survival and therefore contribute to growth of cancer. *Bcl-2* has been identified as an anti-apoptotic gene that is overexpressed in several types of cancer, including colon cancer, thereby preventing cells from initiating apoptosis in response to apoptotic signals (i.e. growth factor depletion or hypoxia) or treatment with chemotherapeutic agents (5, 6). The protein product of the tumor suppressor gene *p53* also plays a vital role in cellular regulation by detecting damaged DNA, inducing G1 arrest, and then initiating either DNA repair or apoptosis to prevent replication of damaged cells. *p53* is mutated or absent in several cancers, including colon cancer, and its loss not only promotes tumor formation, but, because its presence is important to the initiation of apoptosis, its absence attenuates the responsiveness of tumors to radiation and chemotherapeutic intervention (7-9).

Several other oncogenes and tumor suppressor genes have also been identified as being important in the development of colorectal cancer, including the oncogene *Ki-*

ras and the tumor suppressor genes *APC* (adenomatous polyposis coli), *DCC* (deleted in colorectal carcinoma), *MCC* (mutated in colorectal cancer), and the DNA mismatch repair genes, which are involved in HNPCC. *APC* is described as a gatekeeper in the development of colorectal cancer. It is mutated early in many colorectal cancers and this mutation appears to be critical to the initiation and growth of colonic polyps. Later mutations involving other tumor suppressor genes, i.e. *p53*, lead to more advanced stages of tumorigenesis (**Fig. 2-1**) (10).

The APC gene. *APC* is a tumor suppressor gene that maps to the human chromosome region 5q21-q22 (11) and it is widely expressed in normal epithelial tissues (12). Germline mutations in the human *APC* gene are characteristic in persons with FAP, an autosomal dominant inherited disorder resulting in numerous intestinal polyps which develop following somatic mutation of the remaining wild-type *APC* allele (13, 14). However, somatic mutations resulting in loss of full length *APC* protein also occur early in spontaneous forms of the disease (14, 15) indicating an *APC* defect is associated with a majority of human colorectal cancers (16, 17). Furthermore, in the absence of an *APC* mutation, failure to express APC transcripts has been observed in 18 percent of human colonic tumors due to hypermethylation of the promoter region (18). In recent years, several mouse models carrying mutations in murine *Apc* have been developed for the study of intestinal cancer (19-22).

APC encodes a 312 kDa (2,843 amino acids) protein containing a homodimerization domain, seven armadillo repeats of unknown function, and two nuclear export sequences at the NH_2-terminus (**Fig. 2-2**) (23, 24). The C-terminal portion contains a basic domain that binds to microtubules (25), a region interacting with

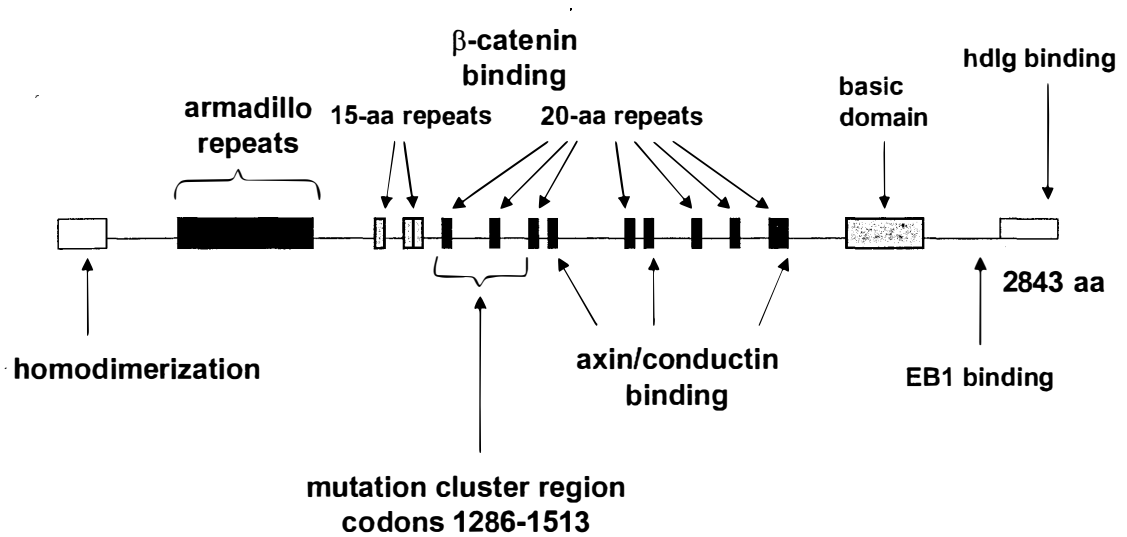

FIGURE 2-2. APC structure and binding domains. Modified from Polakis (23).

EB1 which itself has been shown to associate with microtubules (26), and a domain that binds to the human homolog of the *Drosophila* disc-large protein (DLG) (27). The central region of APC contains three 15-amino acid repeats and seven 20-amino acid repeats, both of which bind β-catenin, the mammalian counterpart to *Drosophila* armadillo (28). Interspersed among the 20-amino acid repeats are three ser-ala-met-pro (SAMP) repeats that mediate interaction with conductin, and inferentially, Axin (29). It is this central region where most mutations occur resulting in a C-terminal truncated protein and tumorigenesis (14).

APC has been shown to act as a tumor suppressor by facilitating the phosphorylation of β-catenin by the serine-threonine kinase glycogen synthase kinase-3β (GSK-3β) (30), which results in the subsequent ubiquitination and proteasomal degradation of β-catenin (31). β-catenin is known to play a role in cell-cell adhesion through interactions with cadherins at adherens junctions and also acts as an important cell signaling molecule in the wnt signaling pathway, which is homologous to wingless signaling in *Drosophila* (**Fig. 2-3**) (32). Wnts are secreted glycoproteins that appear to be involved in differentiation processes. They bind to the seven-transmembrane frizzled receptors, which results in upregulation of β-catenin by preventing formation of the β-catenin destruction complex - a quaternary formation composed of APC, Axin, GSK-3β, and β-catenin - mediated by the cytoplasmic phophoprotein dishevelled, which interacts with Axin to prevent GSK-3β-mediated phosphorylation (33). As cytoplasmic concentrations of β-catenin rise, it migrates to the nucleus where it interacts with Lef/Tcf-4 transcription factors to upregulate transcription of target genes (34), only a few of which have been identified so far including the oncogene *c-MYC*, the cell cycle regulator *cyclin D1*, *PPARδ*, *Tcf1*, *MDR1*, and gastrin (35-40). It has recently been reported that APC

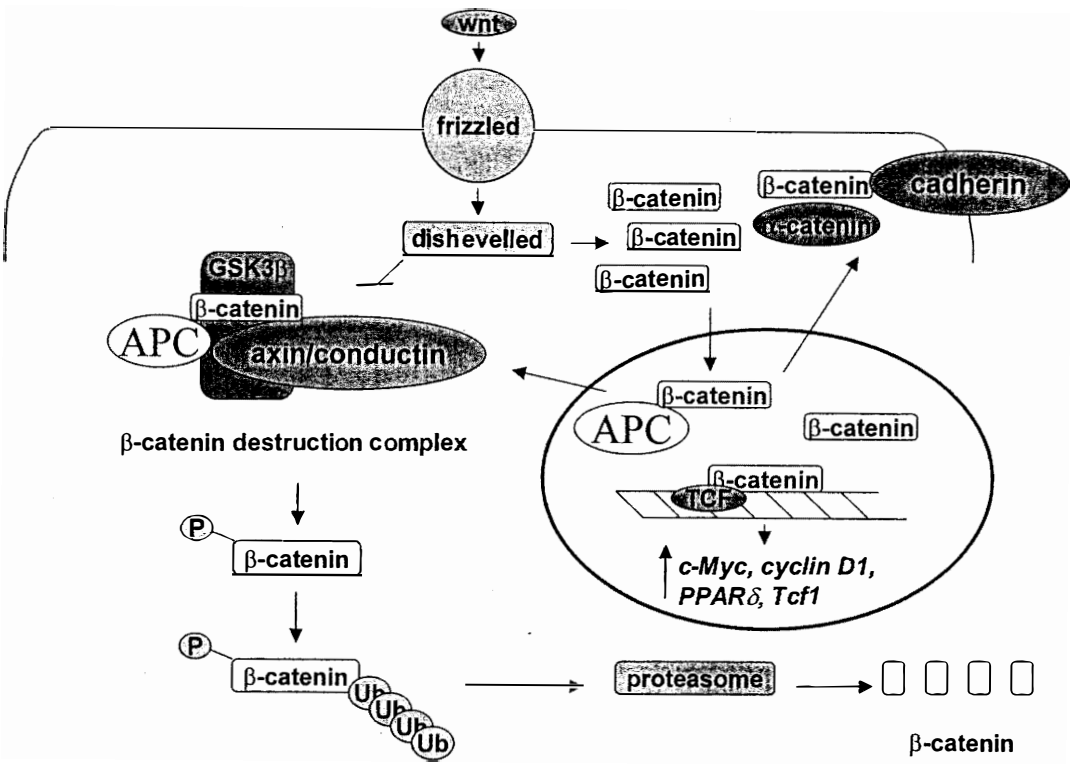

FIGURE 2-3. The Wnt signaling pathway. Modified from Seidensticker and Behrens (32).

contains a nuclear export sequence, suggesting that APC acts primarily as a shuttle to transport β-catenin out of the nucleus and deliver it to the destruction complex, thus preventing β-catenin/Tcf signaling, and also possibly to adherens junctions (24, 41).

Because the carboxy-terminal portion of APC is involved in microtubule assembly and bundling as well as cell migration (25, 42, 43), a truncated APC could conceivably result in intestinal tumorigenesis by impairing the ability of dividing cells to migrate beyond the crypt-villus border. However, the central portion of APC responsible for β-catenin binding has recently been shown to induce growth arrest and apoptosis in colon cancer cells *in vitro* (44). More intriguingly, the presence of at least one SAMP repeat (conferring Axin binding) is sufficient to prevent intestinal tumor formation in mice with a truncating *Apc* mutation at codon 1638 and omission of this axin binding site by truncation at codon 1572 leads to β-catenin accumulation in embryonic stem cells (22). Furthermore, disruption of the Axin binding sites in APC or blocking of the APC binding sites on Axin both prevent APC-induced degradation of β-catenin in human colon cancer cells *in vitro* (45). These data indicate the great importance of Axin/conductin for assembly of the β-catenin destruction complex and the tumor suppressor function of APC. Current evidence suggests Axin acts as a scaffold for formation of the destruction complex (29). Axin itself is phosphorylated by GSK-3β, which increases its stability (46), and then facilitates GSK-3β-mediated phosphorylation of acidic residues on APC, which increases the affinity of APC for β-catenin. APC acts as a shuttle to transport β-catenin to this assembly in which APC binds to Axin, allowing for phosphorylation of β-catenin by GSK-3β (29, 47).

The importance of this pathway in tumorigenesis is strengthened by data showing that 1) β-catenin is frequently dysregulated in colonic tumors expressing wild-type APC

and 2) β-catenin is also elevated in tumors from other sites. For example, colonic tumors from humans and azoxymethane-induced tumors in animal models expressing wild-type APC frequently express β-catenin that is mutated at the GSK-3β phosphorylation sites, which likewise results in stabilization of β-catenin and constitutive activation of target genes (48-51). Furthermore, mutated APC has been identified in tumors at other sites, including the breast (52), and mutated β-catenin has also been identified in human tumors from the liver, colon, ovaries, brain, prostate, uterus, and skin, including desmoid tumors (53-60). For example, a study of 42 desmoid tumors from non-FAP patients found that 22 had β-catenin mutations, 9 had *APC* mutations, and these mutually exclusive mutations resulted in elevated β-catenin protein in all 42 lesions (61). While much of the focus has been on APC and β-catenin, mutations of other components of this signaling pathway could also potentially lead to β-catenin dysregulation. For example, mutations of Axin have been implicated in hepatic tumorigenesis (62). Additionally, overexpression of APC, Axin, or dominant-negative Tcf all downregulate β-catenin and induce cell cycle arrest or apoptosis (34, 49, 51, 62-65).

Familial adenomatous polyposis. FAP is an autosomal dominant disorder present in humans with a germline mutation in *APC* (66) and characterized by development of hundreds to thousands of primarily colorectal adenomatous polyps by the second decade of life. Some of these polyps progress to carcinoma, resulting in a diagnosis of colorectal cancer by 40 years of age. Features of the disease vary by location of the mutation, even within a family. Persons with nonsense or frameshift mutations upstream of codon 157 or downstream of codon 1,600 develop attenuated APC (AAPC) or AFAP, which is a milder form of FAP characterized by fewer tumors and

later onset (67-71). However, most mutations occur between codons 1,000 and 1,600, which has been designated the mutation cluster region (14), and the most severe phenotypes reportedly occur with mutations at codon 1309 (72). Extraintestinal tumors, including retinal lesions, osteomas, brain tumors, desmoid tumors, and cancer of the thyroid, can also occur and their incidence is influenced by location of the germline mutation. However, there is also phenotypic variability with regard to tumor number, location, and extraintestinal tumor incidence within families bearing the same mutation (73). Although this may be due in part to environmental influences such as diet and physical activity, variability may also be due to the presence of gene modifier loci that interact with the *APC* mutation to determine the clinical course.

Apc$^{Min/+}$ mouse model. The *Apc$^{Min/+}$* mouse was discovered following *N*-ethyl-*N*-nitrosurea (ENU) mutagenesis of a C57BL/6J mouse (20). The observed phenotype of multiple intestinal neoplasia (MIN) was suggestive of FAP in humans and subsequent sequence analysis revealed a mutation of the murine *APC* homolog at nucleotide 2549, codon 850, characterized by an A/T –> T/A transversion, resulting in a premature stop codon and a 95 kDa truncated *Apc* gene product. Homozygosity is embryonic lethal, but mice heterozygous for the mutation form multiple adenomas (29±10, by initial report) throughout the intestinal tract, primarily in the small intestine, early in life and rarely live past 150 days of age. As the adenomas do not advance to carcinomas in this model, cause of death is generally due to severe anemia or intestinal obstruction.

Tumor development in *Apc$^{Min/+}$* mice occurs following loss of the remaining wild-type allele (13) associated with loss of the entire chromosome 18, which includes *Apc* (74). Of note, two other genes -- *Mcc* (mutated in colorectal cancer) and *Dcc* (deleted in

colorectal carcinomas) – possibly involved in human colorectal cancer are also present on mouse chromosome 18. Unlike individuals with FAP, *Apc*$^{Min/+}$ mice develop few extraintestinal tumors, with the exception of single mammary adenoacanthomas, which develop in approximately 5% of female *Apc*$^{Min/+}$ mice (75, 76). In addition to the *Apc*$^{Min/+}$ mouse, several other murine *Apc* models have been developed in recent years (**Table 2-1**). These models have been used extensively to investigate the effects of both pharmacological and dietary intervention on *Apc*-mediated intestinal tumorigenesis (77-91).

Phenotypic variation in FAP is related both to the location of the *APC* mutation and the presence of gene modifier loci. The *Apc*$^{Min/+}$ mouse model has been used to identify gene modifier loci that modify neoplastic processes, and crossing *Apc*$^{Min/+}$ mice with the AKR strain was recently shown to reduce intestinal tumor multiplicity to 6.0±4.7 and significantly lengthen the life span (92). Genotyping has revealed a possible modifier locus on mouse chromosome 4, which was designated *Mom1* for "modifier of Min" (93). Secretory phospholipase A_2 maps to the same region as *Mom1* and has been identified as a candidate gene (94). Agreement between the *Pla2g2a* genotype and the *Mom1* phenotype is consistent across several inbred strains. For example, the resistant strains AKR, CAST, and MA have functional *Pla2g2a* alleles and express high levels of *Pla2g2a* mRNA (95). In contrast, B6, the background strain for *Apc*$^{Min/+}$ mice, has an A/T insertion into exon 3 of the *Pla2g2a* allele leading to premature termination, and levels of intestinal *Pla2g2a* mRNA expression are correspondingly low. SWR, DBA2 and BALB strains are *Pla2g2a*+ while 129/Sv-Pas and BTBR carry the same mutation as B6, and the *Mom1* phenotype is concordant among all strains tested (96). This lends support to the proposed role of *Pla2g2a* as modifier of Min phenotype, although it is possible the *Mom1*

TABLE 2-1

Murine Apc models

Model	Mutation and phenotype characteristics	Tumor multiplicity
Apc$^{Min/+}$ (Moser 1990)	Created by ENU-induced germline mutagenesis resulting in a nonsense mutation at codon 850 and truncated Apc protein Tumors develop following loss of chromosome 18, which includes *Apc* *Apc*-/- are embryonic lethal	29±10
Apc$^{\Delta 716}$ (Oshima 1995)	Targeted mutation at codon 716 results in truncated Apc protein *Apc*-/- are embryonic lethal	200-500
Apc^{1638N} (Fodde 1994)	Targeted mutation at codon 1638 results in leaky allele and undetectable levels of truncated Apc protein Tumors show loss of chromosome 18 Mice develop multiple extraintestinal tumors, including desmoids	5-6
Apc^{1638T} (Smits 1999)	Targeted mutation at codon 1638 results in detectable levels of truncated Apc protein Mice do not develop tumors Homozygotes are viable	0

locus could contain multiple genes able to modify tumor multiplicity in *Apc*$^{Min/+}$ mice. While the *Pla2g2c* and *Pla2g5* genes are closely linked to *Pla2g2a* in mice and humans, these have not yet been investigated for correlation to the *Mom1* phenotype. It has been postulated that a FAP modifier locus is located on human chromosome 1p35-36, which is syntenic with *Mom1* in the mouse (97, 98).

DIETARY FAT AND CANCER

Western-style diets characterized by high intakes of energy, fat, meat, and refined grains and sugar, combined with low intakes of fiber, calcium, and fruits and vegetables have been strongly linked to an increased risk of colorectal cancer in epidemiological studies (99-103). Among these dietary factors, the amount and type of dietary fat consumed has been found to be of particular importance (87, 104-107). In animal model studies, high-fat diets consistently elicit higher tumor numbers than low fat diets (87, 106).

Polyunsaturated fatty acids (PUFA) have received much of the attention with regard to the modulation of colorectal cancer by type of dietary fat. PUFA are classified into three main families: n-3, n-6, and n-9, based on the distance of the first double bond from the methyl end of the fatty acid. Linoleic acid (LA, 18:2 n-6) is the parent compound of the n-6 family and it is metabolized through a series of desaturase and elongase steps to arachidonic acid (AA, 20:4 n-6) (**Fig. 2-4**). Likewise, α-linolenic acid (ALA, 18:3 n-3), the parent compound of the n-3 family, competes with n-6 PUFA for desaturase and elongase enzymes to be metabolized to eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) (**Fig. 2-4**). The rate-limiting step in this pathway is the desaturation of LA to γ-linolenic acid (GLA, 20:3 n-6) and of ALA to stearidonic acid

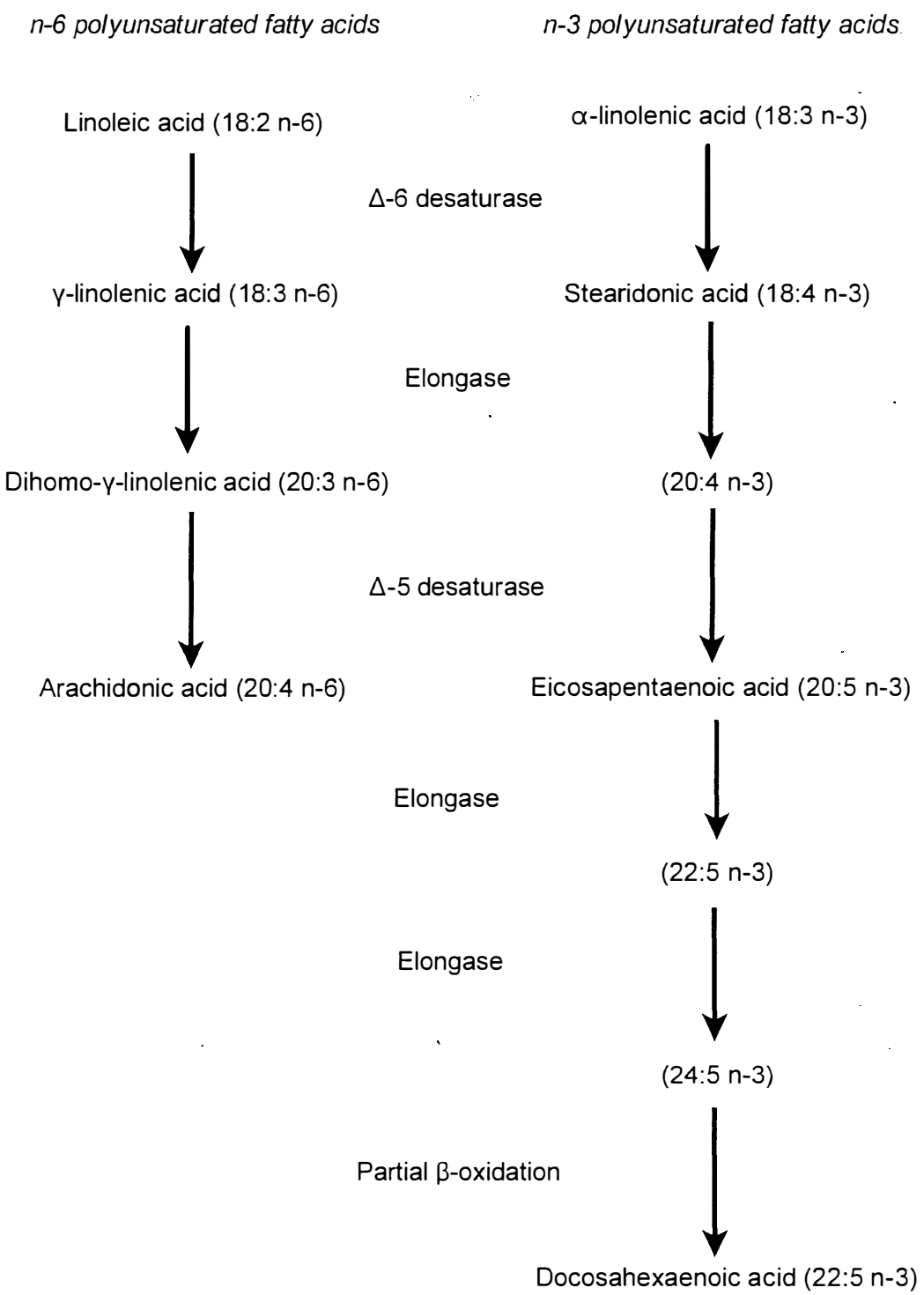

FIGURE 2-4. Pathway of n-6 and n-3 polyunsaturated fatty acid metabolism.

(SDA, 18:3 n-3), respectively, by Δ-6 desaturase (108). LA is by far the predominant PUFA consumed in the U.S. diet at a level of 10-20 g/day, compared to ALA (1-3 g/day) and AA and EPA at levels < 500 mg/day each. As such, LA is the major source of AA present in the tissues, although dietary AA is also a contributor. Corn oil, which contains high levels of LA, has been clearly shown to enhance azoxymethane-induced intestinal tumorigenesis in a rat model, whereas fish oil (as a source of EPA and DHA) inhibits tumorigenesis (106).

n-3 polyunsaturated fatty acids and colon carcinogenesis. Epidemiological evidence links consumption of fish and fish oil, which are rich in EPA and DHA, to a reduced risk of colorectal cancer (99, 104, 107, 109, 110). In an ecological study, Caygill et al. found an inverse relationship between fish and fish oil consumption and colon cancer in 24 European countries (104). While this of itself does not provide conclusive evidence, several large prospective and case-control studies have yielded similar findings (**Table 2-2**) (99, 107, 109, 110). Furthermore, results of several animal studies, conducted primarily in chemically-induced colorectal cancer models, substantiate the epidemiological evidence (**Table 2-3**) (84, 86, 111-115). Recently, two studies in *Apc* mouse models also investigated the effects of more purified forms of n-3 PUFA. Oshima et al. fed $Apc^{\Delta 716}$ mice DHA ethyl ester and observed anti-tumorigenic efficacy in female, but not male, mice (84). Similarly, Paulsen et al. fed $Apc^{Min/+}$ mice n-3 PUFA enriched K-85 fish oil, which contains 54.4% EPA and 30.3% DHA, and observed a dose-dependent decrease in tumors only in female mice and less consistent effects in males (86).

Despite mounting evidence of the protective effect of n-3 PUFA, few studies have utilized purified fatty acids and the mechanisms are not well understood. The prevailing

TABLE 2-2

Epidemiological studies of fish and fish oil intake and intestinal tumorigenesis

	Design	Subjects	Findings
Willett 1990	Prospective cohort (Nurses Health Study)	88,751 women, 34-59 years of age 150 incident cases of colorectal cancer between 1980-1986	χ for Trend: Fish -1.67 (*P*=0.09) Chicken+fish -2.63 (*P*=0.009) Red meat:chicken+fish +3.47 (P=0.0005)
Giovannucci 1994	Prospective cohort (Health Professionals Follow up Study)	47,949 men, 40-75 years of age 205 incident cases of colorectal cancer between 1986-1992	Inverse relationship between fish intake and colon cancer risk (ns)
Caygill 1996	Ecological study	24 European countries, includes both males and females	Fish and fish oil intake have significantly inverse relationship with colorectal cancer mortality rates in both males and females, particularly when expressed in relation to intake of animal fat Fish/animal fat x 100 (Regression coefficient): Males -2.90 (*P*=0.001) Females -1.70 (*P*=0.003)

TABLE 2-2 (continued)

	Design	Subjects	Findings	
Kato 1997	Prospective cohort	14,727 women in New York and Florida, 34-65 years of age 100 incident cases of colorectal cancer between enrollment (1985-1991) and end of follow-up (1994)	Relative risk: Fish+shellfish intake (4^{th} vs. 1^{st} quartile)	0.49 (0.27-0.89)
Fernandez 1999	Case control	Males and females in Italy, <75 years of age Cases (n=828) Controls, unmatched (n=7,990)	Odds ratios: >2 vs. <1 fish servings/wk 1 vs. <1 fish servings/wk	0.6 (0.5-0.7) 0.7 (0.6-0.8)

TABLE 2-3

Animal model studies of n-3 polyunsaturated fatty acids and intestinal tumorigenesis

	Model	Treatment	Effect on tumorigenesis	Comments
Minoura 1988	Azoxymethane-induced tumorigenesis in rat model	EPA 4.7% + LA 0.3% Control: LA 5%	↓ incidence from 69% to 33% ↓ tumor number 1.66±1.69 to 0.41±0.61	EPA, relative to LA, suppressed PGE_2 production
Reddy 1991	Azoxymethane-induced tumorigenesis in rat model	Fish oil 18.5%+5% corn oil Control: 23.5% corn oil Rats were fed diets pre- and post-initiation in a crossover design	Rats fed 23.5% corn oil had 97% tumor incidence Rats fed 18.5% fish oil +5% corn oil had 23% tumor incidence	
Oshima 1996	*Apc*$^{\Delta 716}$ mouse	DHA ethyl ester 3% w/w x 7 wk Control: AIN-93G	Females: ↓ tumor number by 69% Males: no effect	

TABLE 2-3 (continued)

	Model	Treatment	Effect on tumorigenesis	Comments
Paulsen 1997	*Apc*$^{Min/+}$ mouse	K-85 fish oil (54.4% EPA, 30.3% DHA) 0-2.5% (w/w) in the diet replacing corn oil	Females: 48% ↓ in tumors at 2.5% K-85 dose Males: tumors ↓by 66% at 0.4% K-85 dose, NE at higher levels	Variable sample size among groups, n=2 in males receiving K-85 at 2.5% level
Chang 1998	Azoxymethane-induced tumorigenesis in rat model	Fish oil (15% w/w) Control: corn oil (15% w/w)	Adenocarcinoma incidence: Corn oil: 70.3% Fish oil: 56.1%	Fish oil treatment significantly increased apoptosis and differentiation, but had no effect on cell proliferation
Latham 1999	1,2-dimethylhydrazine-induced tumorigenesis in rat model	Fish oil (18.7% EPA, 8% DHA) x 24-48 h post injection of carcinogen Control: corn oil	Significantly fewer ACF developed in fish oil-treated rats	Fish oil-treated rats had higher rates of apoptosis and lower rates of cell proliferation
Boudreau 2001	Athymic nude mice implanted with human colon cancer cells (HCT-116)	Fish oil, 20% (w/w) Control: safflower oil, 20% (w/w)	Fish oil resulted in significantly smaller tumor volumes	

hypothesis, however, is that n-3 PUFA exert their anti-tumorigenic effects through interference with biosynthesis of 2-series prostaglandins from AA (105, 106, 111, 113, 114, 116-121). n-3 PUFA are known to antagonize AA on many levels. They inhibit Δ-6 desaturase (122), and thus inhibit *de novo* AA biosynthesis. They compete for incorporation into cell membrane phospholipids (123). They inhibit expression of cyclooxygenase-2 (COX-2) (124, 125), the committed step in prostaglandin biosynthesis, and also compete for COX activity to minimize formation of the 2-series prostaglandins (123).

However, other mechanisms have also been proposed, including fish oil-mediated alterations in apoptosis and in cell signal transduction via alterations in protein kinase C (PKC) (4, 126), that may or may not be related to prostaglandin signaling. For example, signaling of PGE_2 via the EP1 receptor results in phospholipase C-mediated release of diacylglycerol (DAG) and inositol triphosphate (IP_3), inducing release of intracellular calcium and upregulation of classical PKC subtypes, including PKC-α. PKC-α mRNA and cytosolic protein levels are down-regulated in the colonic mucosa of azoxymethane-treated rats fed fish oil compared to corn oil (126), possibly due to the lower levels of PGE_2 produced following fish oil feeding. Interestingly, other PKC subtypes are affected in an opposite manner, with fish oil increasing expression of PKC-δ and PKC-λ-ζ proteins in the cytosol, while having no effect on levels of membrane-bound protein. These subtypes have been associated with inhibition of cellular proliferation and induction of differentiation (127-129). High-fat fish oil diets (relative to high-fat corn or safflower oil) have also been shown to decrease levels of oncogenic $p21^{ras}$ in colonic and mammary tumors (130, 131).

α-linolenic acid (ALA, 18:3 n-3), the parent fatty acid of the n-3 family, has been studied on a more limited basis than EPA and DHA, but with encouraging results. Dietary perilla oil and flaxseed oil, both rich sources of ALA, have been shown to decrease chemically-induced colonic tumors and aberrant crypt foci (ACF) in a rat model (132-137). These results have coincided with competitive exclusion of n-6 PUFA from membrane phospholipids and associated reductions in PGE_2 concentrations in colonic mucosa (136), suggesting possible prostaglandin involvement.

Stearidonic acid (SDA, 18:4 n-3), the Δ-6 desaturase product of ALA found naturally in blackcurrant (*Ribes nigrum*) seed oil and oils derived from some members of the *Boraginaceae* family (138), has not previously been studied as an anti-tumorigenic agent, although it has been shown to strongly inhibit growth of NIH-3T3 cells (139). The use of dietary SDA as an anti-tumorigenic agent is promising because it is not dependent on the Δ-6 desaturase reaction, the rate-limiting step in *de novo* biosynthesis of long chain PUFA. As such, SDA increases tissue levels of EPA more effectively than does ALA (140, 141). Additionally, other studies suggest that SDA may reduce formation of AA-derived eicosanoids *in vitro* (142, 143). This collective evidence, albeit limited, suggests SDA may have anti-tumorigenic potential.

Anti-tumorigenicity of other polyunsaturated fatty acids. Other fatty acids, including some n-6 PUFA and their derivatives, have also been investigated for purported anti-tumorigenic effects. Conjugated linoleic acid (cLA) refers collectively to several positional and geometric isomers of linoleic acid (LA, 18:2 n-6) in which the double bonds are in conjugation, typically at positions 9 and 11 or 10 and 12 (144). cLA, predominantly as 9(Z),11(E)-18:2 n-7, occurs naturally in small amounts in cooked meats, dairy

products, and ruminant meats (145) and is anti-tumorigenic in chemically-induced rat mammary tumors at levels ≤1 g/100 g diet regardless of the level or type of fat in the base diet, and in murine skin tumors (146-148). It has recently been suggested that cLA exerts its anti-tumorigenic effect by inhibiting metabolism of LA to AA, thereby decreasing biosynthesis of AA-derived prostaglandins (149-152). Compared to studies investigating the efficacy of cLA on mammary tumorigenesis, evidence for protection against colorectal cancer is less definitive. Gavage treatment with cLA has been shown to reduce chemically-induced colonic ACF and gastric neoplasia in mice (153, 154) and cLA treatment inhibited proliferation of human colon tumor cells in vitro (155, 156). However, it has also been reported that dietary cLA has no effect on formation of carcinogen-DNA adducts in colons of rats treated with 2-amino-3-methylimidazo(4,5-*f*)quinoline or 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (157).

γ-linolenic acid (GLA, 18:3 n-6), the Δ-6 desaturase product of LA, is found predominantly in only a few dietary sources including evening primrose (*Oenothera biennis*) oil, borage (*Boraginaceae*) oil, blackcurrant seed oil, and spirulina (138). By bypassing the rate-limiting Δ-6 desaturase reaction, dietary GLA is rapidly metabolized to dihomo-γ-linolenic acid (DGLA, 20:3 n-6). As a 20-carbon PUFA, DGLA purportedly competes with AA for COX and 15-lipoxygenase activity to produce PGE_1 and 15-hydroxyeicosatrienoic acid (15-HETrE), respectively, in some cell types, and may thereby attenuate formation of AA-derived metabolites (158-161). A second proposed mechanism whereby GLA might elicit anti-tumorigenic effects is via increased lipid peroxidation (162-165). Regardless, the actual anti-tumorigenicity of GLA has been only narrowly investigated thus far. GLA treatment has been shown to decrease metastatic potential of human colon cancer cell lines and block cell cycle progression in vitro (166-

175). Accordingly, arterial GLA injections have been shown to reduce growth of implanted hepatoma cells in rats (164) and rabbits (176), while intra-tumoral injections of Lithium-GLA reduced the volume of implanted pancreatic tumor cells in mice (177). However, dietary GLA has demonstrated potential efficacy as an anti-tumorigenic agent only in 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary tumors in rats, a study in which a diet containing 20% evening primrose oil reduced tumor incidence compared to 20% corn oil (178), and in nude mice bearing breast carcinoma xenografts (179). A preventive role for dietary GLA in gastrointestinal tumorigenesis has not previously been established.

PROSTAGLANDINS AND INTESTINAL TUMORIGENESIS

Arachidonic acid is present in the American diet and is consumed in relatively small quantities of ~200 mg/day from animal sources (180). Alternatively, arachidonic acid can be synthesized from linoleic acid through a series of elongations and desaturations, as described previously. It is readily incorporated into membrane phospholipids almost exclusively at the sn-2 position. Arachidonic acid release is mediated by phospholipases and free arachidonic acid can then be acted upon by cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 oxygenases to form a multitude of bioactive eicosanoids. Prostaglandin E_2 (PGE_2), the COX product of arachidonic acid metabolism, is present at high levels in intestinal tumors from humans and *Apc*$^{Min/+}$ mice (77, 181, 182). It has proliferative effects *in vivo* and *in vitro* and is therefore thought to play a role in promotion of tumor growth.

E prostaglandin receptors. Prostaglandins act through their respective receptors to initiate intracellular signaling cacades and evoke cellular responses. PGE_2,

as well as other E prostaglandins, bind to the E prostaglandin (EP) receptors, of which four subtypes have been identified (EP1-EP4) (**Table 2-4**). These are seven-transmembrane spanning, G protein-coupled rhodopsin-type receptors with differential signaling mechanisms, tissue expression patterns, and roles that are still being elucidated. Signaling through EP1 results in mobilization of intracellular calcium as well as capacitative calcium entry, which culminates in a rise in intracellular calcium levels (183, 184). EP2, EP3, and EP4 act primarily through cAMP-mediated signaling pathways. Both EP2 and EP4 signal through G_s to stimulate adenylate cyclase, increase cAMP and increase phosphorylation of target proteins by the serine-threonine kinase protein kinase A (PKA). EP3 is the most well-characterized of the EP receptors and of its three splice variants, α and β inhibit adenylate cyclase, while γ is generally inhibitory in the presence of low ligand concentrations and stimulatory in the presence of high ligand concentrations. Evidence also indicates that EP3 has the ability to increase IP_3 and intracellular calcium levels via mobilization of intracellular calcium stores and capacitative calcium entry (185). EP1 and EP4 have been considered the most important EP receptor subtypes in the gastrointestinal tract, although EP3 is also expressed (186). In a recent experiment, EP1 knockout mice developed significantly fewer ACF following azoxymethane treatment and *Apc*$^{Min/+}$ mice treated with a selective EP1 antagonist developed 44% fewer tumors compared to untreated controls (89). In contrast, mice lacking EP3 were not protected from developing ACF, indicating that EP receptors have differential effects on intestinal tumorigenesis and that EP1 may be the most important of the EP receptors in this regard.

Cyclooxygenase, the committed step in prostaglandin biosynthesis. Two isoforms of COX, also known as prostaglandin H_2 synthase (PGHS), have been identified

TABLE 2-4

E prostaglandin (EP) receptors

Receptor subtype	Signaling	Murine intestinal expression	Role in intestinal tumorigenesis
EP1	G_s: phospholipase C, ↑ mobilization of intracellular calcium and capacitative calcium entry	Smooth muscle cells of muscularis mucosa throughout gastrointestinal tract	EP1-/- mice developed significantly fewer azoxymethane-induced ACF than normal controls EP1 receptor antagonist decreases tumors by 44% in *Apc*$^{Min/+}$ mice
EP2	G_s: ↑cAMP	Not reported in normal murine intestine	None known
EP3 α,β,γ	G_i: ↓ cAMP (α,β) $G_i/_s$: ↓/↑ cAMP dependent upon PGE concentration (γ) G_i: ↑ phospholipase C, ↑ mobilization of intracellular calcium and capacitative calcium entry	Strongly expressed in myenteric ganglia, weakly expressed in longitudinal smooth muscle throughout murine intestinal tract	None known EP3-/- mice did not differ from controls in development of azoxymethane-induced ACF
EP4	G_s: ↑cAMP	Localized to intestinal epithelial cells in upper portion of villi	None known

thus far and they have been designated COX-1 and COX-2. Although encoded by two distinct genes, bearing only 60% identity, and having distinctly different patterns of expression, they both metabolize arachidonic acid in the same manner. In a two-step process, arachidonic acid is first oxidized (cyclooxygenated) to prostaglandin G_2 (PGG_2) and the endoperoxide moiety subsequently reduces PGG_2 to prostaglandin H_2 (PGH_2), which in turn is acted upon by additional enzymes to form the biologically-active prostanoids including PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and TXA_2 (**Fig. 2-5**) (187, 188). COX-1 is a 68.7 kDa protein that is constitutively expressed at low levels in most tissues and is associated with membranes of the endoplasmic reticulum (ER) and nuclear envelope. COX-2 is a 69.0 kDa protein localized also to the nuclear envelope and ER, but is much more concentrated than COX-1 in the nuclear envelope (189). COX-2 is the inducible isoform expressed in response to pro-inflammatory or mitogenic stimuli, hormones, and growth factors such as EGF (190). Prostaglandins synthesized by COX are known to be cytoprotective in the stomach and intestinal tract, thus COX inhibition via nonsteroidal anti-inflammatory drugs (NSAIDs) leads to mucosal damage and ulceration. Prostaglandins also play important roles in kidney and platelet function, gestation and parturition. In addition to these roles, COX-2 also plays roles in nerve transmission, febrile response, and hyperalgesia.

In recent years, several lines of evidence have linked COX to intestinal cancer both in humans and in animal models. First, COX-2 is expressed in tumors, but not normal intestinal tissue, from humans with FAP or sporadic colorectal cancer (191-193) as well as from *Apc*$^{Min/+}$ mice (194, 195), where it is localized predominantly in interstitial macrophages (192, 195). COX-2 overexpression is also associated with tumors at other sites including human cancers of the lung, breast, skin, and others (196-199). COX-2

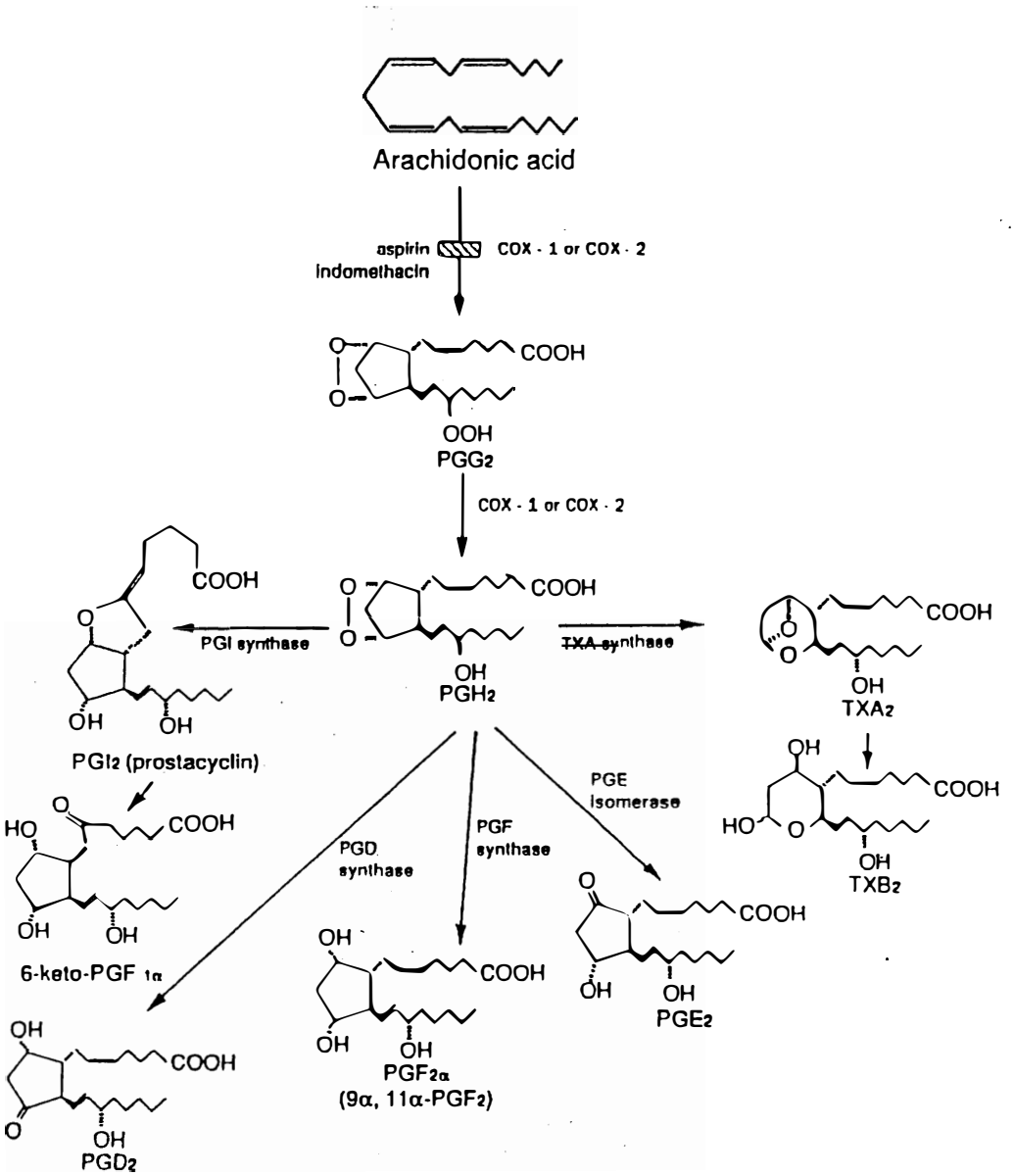

FIGURE 2-5. Metabolism of arachidonic acid via the cyclooxygenase pathway from Vane et al. (188).

was recently found to be upregulated by Wnt-3 or inhibition of GSK-3β in mouse mammary epithelial cells, but these effects were independent of β-catenin, suggesting COX-2 is not a target of β-catenin/Tcf signaling and that Wnt signaling is not dependent upon β-catenin (200). Furthermore, crossing *Apc* knockout mice ($Apc^{\Delta 716}$) with COX-2 knockout mice dose-dependently reduced tumor number by 34% and 86% (201) and crossing $Apc^{Min/+}$ mice with COX-2 or COX-1 knockout mice reduced tumor number by 84% and 77%, respectively (202). Finally, NSAIDs, inhibitors of COX, reduce tumorigenesis in both humans and animal models.

Nonsteroidal anti-inflammatory drugs as anti-tumorigenic agents. The NSAIDs are classified according to their binding characteristics with COX as follows: Class I compounds such as piroxicam, sulindac sulfide, and ibuprofen are simple competitive inhibitors. Class II compounds including indomethacin, flurbiprofen, and meclofenamate, are competitive, slow reversible inhibitors. Class III compounds, most notably aspirin, are competitive irreversible inhibitors which covalently modify the COX active site by acetylating a serine residue. The above-listed NSAIDs are non-selective COX inhibitors, meaning they inhibit both COX-1 and COX-2 (203). Selective COX-2 inhibitors have been developed in recent years with the purpose of preventing prostaglandin formation associated with pathological conditions such as arthritis, while circumventing the deleterious side-effects associated with non-selective inhibitors. COX-1 is considered the isoform responsible for "housekeeping" functions such as maintenance of gastric and intestinal mucosa and, accordingly, it has been thought that COX inhibition with non-selective inhibitors is ulcerogenic due only to inhibition of COX-1 (204). However, it was recently demonstrated that both COX-1 and COX-2 are important

in this regard (205). Both non-selective and selective COX-2 inhibitors have been investigated for potential anti-tumorigenic capabilities in humans and animal models.

Animal studies. The most studied NSAIDs in animal models of intestinal tumorigenesis are sulindac and piroxicam. Long-term treatment with either of these non-selective NSAIDs results in development of up to 99% fewer tumors in $Apc^{Min/+}$ mice (77, 82) (78, 80). Most striking, however, is the ability of these non-selective NSAIDs to induce regression of 75-90% of preexisting tumors following only 4-6 days of treatment (77, 80). Indomethacin is also anti-tumorigenic in this model (90). Although aspirin treatment has been reported to inhibit tumorigenesis by up to 44-55% (81, 206), studies from our laboratory have shown no effect (90). The effects of aspirin are unique among NSAIDs in that acetylation of the serine residue on COX-1 inactivates the enzyme, but acetylation of COX-2 serine 516 does not. Rather, this acetylation causes COX-2 to produce a different metabolite --15*R*-HETE (207). Selective COX-2 inhibitors have also been shown to inhibit tumorigenesis in $Apc^{Min/+}$ mice, but with lesser efficacy than the non-selective inhibitors, resulting in 52-71% fewer tumors in $Apc^{Min/+}$ mice relative to untreated controls (79, 88).

Epidemiology. Consistent with animal data regarding NSAIDs, epidemiological evidence from several large cohorts has suggested a possible chemopreventive effect of aspirin in humans (208-210). However, results of the first randomized trial and prospective cohort study of aspirin use and sporadic colorectal cancer challenged these findings (211). The Physician's Health Study followed 22,071 U.S. male physicians who were randomized to receive 325 mg of aspirin or placebo every other day for five years,

after which time they were permitted to self-select aspirin or placebo for one year. Thirteen years after the study began, there was no difference in the incidence of colorectal cancer based on aspirin use (211), suggesting confounding variables may have accounted for the inverse relationship reported from the earlier observational studies. Randomized trials of non-aspirin NSAIDs have been more convincing.

Human trials. A few randomized controlled trials in humans have evaluated the efficacy of sulindac specifically and found that it effectively regressed polyps in persons with FAP over a period of a few months (212-214), and regression was associated with a higher apoptotic ratio than in placebo-treated controls (214). However, sulindac therapy was less effective following long-term treatment (212, 213, 215). For example, in an open study of 15 FAP patients, sulindac treatment resulted in significant regression by the 6 month time point, but there was no significant difference from baseline by the end of the study (12-124 months) (215), suggesting that sulindac-resistant tumors develop over time. The selective COX-2 inhibitor celecoxib was also shown to be anti-tumorigenic in FAP during short-term treatment, reducing polyp burden by 30.7% compared to placebo (216). The effects of long-term selective COX-2 inhibition have not been reported.

Evidence for cyclooxygenase-independent mechanisms. Despite an accumulation of supportive data on many fronts, mounting evidence suggests that NSAIDs may work via both COX-dependent and COX-independent mechanisms. For example, *S*-flurbiprofen, a nonselective COX inhibitor, and its inactive enantiomer (*R*-flurbiprofen) were equally effective in reducing tumor number in $Apc^{Min/+}$ mice (217).

Sulindac sulfone, a metabolite of sulindac lacking COX-inhibitory activity, has been shown to inhibit chemically-induced colon carcinogenesis in rats while having no effect on prostaglandin levels (218). Studies of sulindac sulfone have also demonstrated a trend toward increasing apoptosis in polyps of FAP patients, although a decrease in polyp number was not observed (219). Additionally, NSAIDs have been shown to modulate cell proliferation and cell death in colon cancer cells lacking COX and the addition of exogenous prostaglandins did not reverse these effects, suggesting that not all NSAID effects are based on COX inhibition (220-223). Multiple COX-independent mechanisms have been investigated to date including modulation of angiogenesis (224-226), phospholipase A_2 ($cPLA_2$) (227-229), 15-lipoxygenase-1 and consequent 13-*S*-hydroxyoctadecadienoic acid (13-*S*-HODE) production (230), ceramide (231-233), inducible nitric oxide synthase (iNOS) (234-236), p21 (237), β-catenin (238)(222, 239, 240), peroxisome proliferator-activated receptors (PPARs) (241-246), IκB kinase (247-249), Akt (250, 251), bcl-2 (238), and cGMP phosphodiesterase (252) (**Table 2-5**). While many of these alternative mechanisms may involve signaling pathways related to COX inhibition and prostaglandin biosynthesis, these links have yet to be definitively established.

TABLE 2-5

Investigations of cyclooxygenase-independent mechanisms for the anti-tumorigenic effects of NSAIDs

Mechanism	Supportive studies	Findings
Akt	Hsu 2000 (251)	Selective COX-2 inhibitor celecoxib decreased activation of Akt and increased apoptosis in prostate cancer cell lines
	Fukumoto 2001 (250)	Akt is activated by Wnt/Dishevelled, binds to Axin/GSK-3β and increases levels of free β-catenin to upregulate the Wnt signaling pathway
Angiogenesis	Masferrer 2000 (226)	Selective COX-2 inhibitor celecoxib decreased angiogenesis in rat corneal model
	Williams 2000 (224)	Implanted tumors in COX-2-/- mice less well-vascularized than in wild-type mice. COX-2-/- murine fibroblasts and wild-type fibroblasts treated with a selective COX-2 inhibitor had >90% reduction in vascular endothelial growth factor (VEGF) production
	Brekken 2000 (225)	Anti-VEGF antibody inhibited tumor growth in nude mice
β-catenin	Mahmoud 1998 (81)	Aspirin decreased intracellular β-catenin levels in enterocytes of *Apc*$^{Min/+}$ mice
	McEntee 1999 (238)	Sulindac decreased intracellular β-catenin levels in tumors of *Apc*$^{Min/+}$ mice
	Smith 2000 (222)	Indomethacin decreased intracellular β-catenin levels in several colon cancer cell lines, while aspirin and the selective COX-2 inhibitor NS-398 did not

TABLE 2-5 (continued)

Mechanism	Supportive studies	Findings
β-catenin (continued)	Dihlmann 2001 (240)	Aspirin and indomethacin inhibit transcription of β-catenin/TCF target genes in colon tumor cells without modulating β-catenin levels or complexing with TCF
Bcl-2	McEntee 1999 (238)	Sulindac decreased levels of Bcl-2 in tumors of $Apc^{Min/+}$ mice
$cPLA_2$	Yuan 2000 (227)	Treatment of NIH-3T3 cells with sulindac or aspirin suppressed $cPLA_2$ expression, with sulindac treatment having a much more pronounced effect, suggesting NSAIDs inhibit COX, but also limit AA availability as COX substrate
	Takaku 2000 (228)	$cPLA_2$ is overexpressed in polyps of $Apc^{\Delta 716}$ mice, introduction of a genetic mutation into the $cPLA_2$ gene significantly reduced polyp size, but not number
	Hong 2001 (229)	$Apc^{Min/+}$, $cPLA_2$-/- mice had 83% fewer tumors than $Apc^{Min/+}$ littermates
cGMP phosphodiesterase	Thompson 2000 (252)	Sulindac sulfone inhibits cGMP phosphodiesterase, for which β-catenin is a substrate, in the colon cancer cell line SW480
iNOS	Rao 1999 (236)	iNOS increases COX-2 expression, prostaglandin production. A selective iNOS inhibitor inhibited ACF development by 58% in rats treated with azoxymethane while the non-specific iNOS inhibitor curcumin inhibited ACF development by 45%
	Kawamori 2000 (234)	The NOS inhibitor L-N(G)-nitroarginine methyl ester also inhibited azoxymethane-induced ACF development in rats

TABLE 2-5 (continued)

Mechanism	Supportive studies	Findings
iNOS (continued)	Mei 2000 (235)	Nitric oxide increases cytoplasmic levels of β-catenin and promotes formation of β-catenin/LEF-1 complexes
NF-κB	Yamamoto 1999 (247)	Aspirin, sulindac sulfide and sulindac sulfone inhibit IκB kinase β (IKKβ), which is necessary for the activation of NF-κB, in colon cancer cell lines and others *in vitro*
	Rossi 2000 (248)	Anti-inflammatory cyclopentanone prostaglandins (i.e. 15-deoxy-Δ-$^{12\text{-}14}$-PGJ_2) inhibit IKKβ *in vitro*
p21	Patrignani 1999 (237)	The selective COX-2 inhibitor L-745,337 dose-dependently induced $p21^{WAF\text{-}1/cip1}$ in HT-29 colon cancer cells *in vitro*, aspirin and sodium salicylate had no effect
	Yang 2001 (249)	Inactivation of $p21^{WAF\text{-}1/cip1}$ increases number and size of tumors in Apc^{1638} mice
PPARs	Saez 1998 (243), LeFebvre 1998 (244)	Troglitazone, a PPARγ activator, increased the number of colonic tumors in $Apc^{Min/+}$ mice in two separate studies
	Sarraf 1998 (245)	Growth of human colon cancer cell xenografts in mice was inhibited by troglitazone
	He 1999 (35)	PPARδ is a target of β-catenin/TCF signaling

TABLE 2-5 (continued)

Mechanism	Supportive studies	Findings
PPARs (continued)	Park 2001 (246)	PPARδ null cells xenografted into nude mice were less able to form tumors than PPARδ+/- or wild-type controls
13-*S*-HODE	Shureiqi 2000 (230)	15-lipoxygenase-1 is upregulated by NSAIDs (sulindac sulfone, selective COX-2 inhibitor NS-398) resulting in formation of the linoleic acid product 13-*S*-HODE; inhibiting 15-lipoxygenase-1 blocked NSAID-induced apoptosis in a COX-2 -/- colon cancer cell line
Sphingolipids	Chan 1998 (222)	Sulindac sulfide treatment of colon cancer cells *in vitro* significantly increased AA and ceramide levels and this was associated with apoptotic cell death
	Schmelz 1999 (232)	Dietary ceramide-beta-D-glucuronide significantly suppressed ACF formation and cell proliferation in 1,2-dimethylhydrazine-treated CF1 mice
	Schmelz 2000 (233)	Dairy glycosphingolipids reduced ACF formation and cell proliferation in 1,2-dimethylhydrazine-treated CF1 mice

RESEARCH OBJECTIVES

The overall objective of this research was to more clearly establish the link between dietary fatty acids and tumorigenesis. This objective was achieved by addressing the following specific aims:

1) Determine if select dietary n-3 and n-6 polyunsaturated fatty acids (PUFA) will reduce intestinal tumorigenesis in the *Apc*$^{Min/+}$ mouse model and, if so, determine which are most efficacious.

2) Determine if altering arachidonic acid levels and/or its metabolism by dietary fatty acids can modulate intestinal tumorigenesis in the *Apc*$^{Min/+}$ mouse model.

3) Determine if selective inhibition of de novo biosynthesis of arachidonic acid affects tumorigenesis in the *Apc*$^{Min/+}$ mouse model.

4) Determine if prostaglandins are mediators of intestinal tumorigenesis.

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PART III

Highly-unsaturated n-3 Fatty Acids, but not α-Linolenic, Conjugated Linoleic or γ-Linolenic Acids, Reduce Tumorigenesis in *Apc*$^{Min/+}$ Mice

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ABSTRACT

We showed previously that dietary eicosapentaenoic acid (EPA, 20:5 n-3) is anti-tumorigenic in the *Apc*$^{Min/+}$ mouse, a genetic model of intestinal tumorigenesis. Only a few studies have evaluated the effects of dietary fatty acids, including EPA and docosahexaenoic acid (DHA, 22:6 n-3), in this animal model and none have evaluated the previously touted anti-tumorigenicity of α-linolenic acid (ALA, 18:3 n-3), conjugated linoleic acid (cLA, 18:2 n-7), or γ-linolenic acid (GLA, 18:3 n-6). Stearidonic acid (SDA, 18:4 n-3), the Δ-6 desaturase product of ALA, which is readily metabolized to EPA, has not been previously evaluated for anti-tumorigenic efficacy. This study was undertaken to evaluate the anti-tumorigenicity of these dietary fatty acids (ALA, SDA, EPA, DHA, cLA and GLA) compared with oleic acid (18:1 n-9) at a level of 3 g/100 g in the diets of *Apc*$^{Min/+}$ mice and to determine whether any alterations in tumorigenesis correspond to alterations in prostaglandin biosynthesis. Tumor multiplicity was reduced by ~50% in mice fed SDA or EPA compared with controls, whereas less pronounced effects were observed in mice fed DHA (P = 0.15). ALA, cLA, and GLA were ineffective at the dose tested. Although lower tumor numbers coincided with significantly lower prostaglandin levels in SDA- and EPA-fed mice, ALA and DHA supplementation resulted in equally low prostaglandin levels, despite proving less efficacious with regard to tumor number. Prostaglandin levels did not differ significantly in the cLA and GLA groups compared with controls. These results suggest that SDA and EPA attenuate tumorigenesis in this model and that this effect may be related in part to alterations in prostaglandin biosynthesis.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer deaths in the United States with 56,503 deaths reported in 1997 (1). Several lines of evidence overwhelmingly implicate environmental factors, specifically dietary components, as major variables influencing colorectal cancer incidence. Western-style diets characterized by high intakes of energy, fat, meat, refined grains and sugar, combined with low intakes of fiber, calcium, and fruits and vegetables have been strongly linked to an increased risk of colorectal cancer (2-4). Among these, the amount and type of dietary fat consumed is of particular importance (5-8).

Epidemiological studies indicate that consumption of fish and fish oil correlates with a reduced risk of colorectal cancer (5, 9, 10). Fish oil is rich in the n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Studies in humans and in chemically-induced colonic tumor animal models overwhelmingly indicate a protective effect of n-3 PUFA and the mechanism is largely thought to be related to interference with biosynthesis of 2-series prostaglandins from arachidonic acid (AA, 20:4 n-6) (11-15). We and others demonstrated recently that diets containing EPA, and possibly DHA, are also anti-tumorigenic in a murine model of human colorectal cancer and this effect is related, at least in part, to interference with AA metabolism (6, 16, 17). α-linolenic acid (ALA, 18:3 n-3), the parent fatty acid of the n-3 family, has been studied on a more limited basis, but with some promising results. Dietary perilla oil and flaxseed oil, both rich sources of ALA, have been shown to decrease chemically-induced colonic tumors and aberrant crypt foci (ACF) in a rat model (18-21). These results have coincided with competitive exclusion of n-6 PUFA from membrane phospholipids and associated reductions in

PGE_2 concentrations in colonic mucosa (20). Stearidonic acid (SDA, 18:4 n-3), the Δ-6 desaturase product of ALA found naturally in blackcurrant (*Ribes nigrum*) seed oil and oils derived from some members of the *Boraginaceae* family (22), has not been studied previously as an anti-tumorigenic agent, although it has been shown to strongly inhibit growth of NIH-3T3 cells (23). The use of dietary SDA as an anti-tumorigenic agent is promising because it is not dependent on the Δ-6 desaturase reaction, the rate-limiting step in de novo biosynthesis of long chain PUFA. As such, SDA increases tissue levels of EPA more effectively than does ALA (24, 25). Furthermore, other studies suggest that SDA may reduce formation of AA-derived eicosanoids *in vitro* (26, 27). This collective evidence, albeit limited, warrants investigation into the anti-tumorigenic potential of SDA.

Other fatty acids, including some n-6 PUFA and their derivatives, have also been investigated for purported anti-tumorigenic effects. Conjugated linoleic acid (cLA) refers collectively to several positional and geometric isomers of linoleic acid (LA, 18:2 n-6) in which the double bonds are in conjugation, typically at positions 9 and 11 or 10 and 12 (28). cLA, predominantly as 9(Z),11(E)-18:2 n-7, occurs naturally in small amounts in cooked meats, dairy products, and ruminant meats (29) and is potently anti-tumorigenic in chemically-induced rat mammary tumors and murine skin tumors (30, 31). It has recently been suggested that cLA exerts its anti-tumorigenic effect by inhibiting metabolism of LA to AA, thereby decreasing biosynthesis of AA-derived prostaglandins (32-34). Compared with studies investigating the efficacy of cLA on mammary tumorigenesis, evidence for protection against colorectal cancer is less definitive. To date, gavage treatment with cLA has been shown to result in fewer chemically-induced colonic ACF and lessened gastric neoplasia in mice (35, 36) and cLA treatment reduced proliferation of human colon tumor cells in vitro (37, 38).

γ-linolenic acid (GLA, 18:3 n-6), the Δ-6 desaturase product of LA, is found predominantly in only a few dietary sources including evening primrose (*Oenothera biennis*) oil, borage (*Boraginaceae*) oil, blackcurrant seed oil, and spirulina (22). By bypassing the rate-limiting Δ-6 desaturase reaction, dietary GLA is rapidly metabolized to dihomo-γ-linolenic acid (DGLA, 20:3 n-6). As a 20-carbon PUFA, DGLA purportedly competes with AA for cyclooxygenase (COX) and 15-lipoxygenase activity to produce PGE_1 and 15-hydroxyeicosatrienoic acid (15-HETrE), respectively, in some cell types, and may thereby attenuate formation of AA-derived metabolites (39-41). A second proposed mechanism whereby GLA might elicit anti-tumorigenic effects is via increased lipid peroxidation (42-44). Nevertheless, the actual anti-tumorigenicity of GLA has been only narrowly investigated thus far. GLA treatment has been shown to decrease metastatic potential of human colon cancer cell lines and block cell cycle progression *in vitro* (45, 46). Accordingly, arterial GLA injections have been shown to inhibit growth of implanted hepatoma cells in rats (43) and intratumoral injections of lithium-GLA resulted in smaller tumor volumes in mice implanted with pancreatic tumor cells (47). However, dietary GLA has demonstrated potential efficacy as an anti-tumorigenic agent only in 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary tumors in rats, a study in which a diet containing evening primrose oil (20 g/100 g) resulted in a lower tumor incidence than did corn oil (48), and in nude mice bearing breast carcinoma xenografts (49). A preventive role for dietary GLA in gastrointestinal tumorigenesis has yet to be established.

Over the last several years, the $Apc^{Min/+}$ mouse model has been used to evaluate the effects of nutritional intervention on intestinal tumorigenesis because of its germline mutation in the murine adenomatous polyposis coli (*Apc*) gene (6, 16, 17, 50). After

somatic mutation of the wild-type allele, these mice spontaneously develop adenomas throughout the intestinal tract with preferred localization in the small intestine (51). Development of colorectal cancer in humans from dysplastic crypts to metastatic carcinoma involves a series of genetic mutations, the earliest often involving *APC* (52, 53). Individuals with familial adenomatous polyposis (FAP), like $Apc^{Min/+}$ mice, possess a germline mutation in *APC*, and mutational damage or loss of the wild-type allele initiates intestinal tumor formation (54, 55). Although FAP accounts for <1% of all human colorectal cancer cases, somatic mutations resulting in loss of full length *APC* protein also occur early in spontaneous forms of the disease (53, 55) indicating an *APC* defect is associated with a majority of human colorectal cancers (56, 57). Therefore, in the present study, we investigated effects of the dietary fatty acids ALA, SDA, EPA, DHA, cLA, and GLA on intestinal tumorigenesis in $Apc^{Min/+}$ mice fed diets based on a typical Western diet.

MATERIALS AND METHODS

Animals. Male C57BL/6J $Apc^{Min/+}$ mice (n=77; 37 d old) (Jackson Laboratories, Bar Harbor, ME), were randomized into eight dietary groups (n = 9-10 mice/group) on arrival. They were housed in a temperature-controlled room with 12-h light:dark cycle and given free access to food and water. The health of the mice was checked daily. Food was withheld from them overnight before they were killed. All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Diets. All diets were based on the composition of a typical Western diet with the following energy distribution: carbohydrate, 43.4%; fat, 35.4%; and protein, 21.2%. These diets were predicated on the "US17" diet formulated by Monsanto Co. (St. Louis, MO) and Research Diets, Inc. (New Brunswick, NJ) with input from the authors. The following fatty acid ethyl esters were added (31 g/kg) to the base diet at the expense of Trisun high-oleic acid (OA, 18:1 n-9) sunflower oil (Monsanto Co., St. Louis, MO): 1) conjugated linoleic acid (cLA, 9,11-18:2 n-7 isomers, 77%; 10,12-18:2 n-6, 13%; mixed isomers, 5%), 2) gamma-linolenic acid (GLA, 18:3 n-6, 95% pure), 3) α-linolenic acid (ALA, 18:3 n-3, 95% pure), 4) stearidonic acid (SDA, 18:4 n-3, 85% pure), eicosapentaenoic acid (EPA, 20:5 n-3, 95% pure), or docosahexaenoic acid (DHA, 22:6 n-3, 90% pure). The diet containing the nonsteroidal anti-inflammatory drug (NSAID) sulindac (320 mg/kg) was used as a positive control **(Table 3-1)**. Overall fatty acid compositions of the diets are shown in **Table 3-2**. All diets were divided into daily aliquots and stored under an atmosphere of nitrogen at -80°C to prevent oxidation. All mice were provided fresh food daily. Body weights were recorded weekly.

Experimental design. After randomization into dietary groups, all animals were maintained on assigned diets for ~7 wk. At 87-89 d of age, they were killed by cervical dislocation and tumor number, size, and location were determined as previously described (51). Portions of normal-appearing intestine and several of the largest tumors were harvested for histological examination based on previously reported features of intestinal development and dysplasia in this strain of mice. Phospholipid fatty acid composition and basal levels of PGE_2 and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) were determined for each mouse from normal-appearing jejunal sections of the small intestine.

TABLE 3-1

Experimental diet composition[1]

	Dietary groups							
					Omega-3 fatty acids			
Component	Control	Sulindac	cLA	GLA	ALA	SDA	EPA	DHA
				g/kg				
Casein	229	229	229	229	229	229	229	229
L-cystine	3	3	3	3	3	3	3	3
Corn starch	274	274	274	274	274	274	274	274
Maltodextrin 10	86	86	86	86	86	86	86	86
Sucrose	114	114	114	114	114	114	114	114
Cellulose	57	57	57	57	57	57	57	57
Cocoa Butter	43	43	43	43	43	43	43	43
Linseed oil	5	5	5	5	5	5	5	5
Palm oil	60	60	60	60	60	60	60	60

TABLE 3-1 (continued)

	Control	Sulindac	cLA	GLA	ALA	SDA	EPA	DHA
Safflower oil	33	33	33	33	33	33	33	33
Sunflower oil, Trisun[2]	31	31	0	0	0	0	0	0
9(Z),11(E)-18:2 n-7	0	0	31	0	0	0	0	0
18:3 n-6 ethyl ester	0	0	0	31	0	0	0	0
18:3 n-3 ethyl ester	0	0	0	0	31	0	0	0
18:4 n-3 ethyl ester	0	0	0	0	0	31	0	0
20:5 n-3 ethyl ester	0	0	0	0	0	0	31	0
22:6 n-3 ethyl ester	0	0	0	0	0	0	0	31
Sulindac	0	.32	0	0	0	0	0	0
Salt mix RD-96[3]	11	11	11	11	11	11	11	11
Di calcium phosphate	15	15	15	15	15	15	15	15
Calcium carbonate	6	6	6	6	6	6	6	6
Potassium citrate	19	19	19	19	19	19	19	19
Vitamin mix V13401[4]	11	11	11	11	11	11	11	11
Choline bitartrate	2	2	2	2	2	2	2	2
alpha vitamin E acetate	.15	.15	.15	.15	.15	.15	.15	.15

TABLE 3-1 (continued)

	Control	Sulindac	cLA	GLA	ALA	SDA	EPA	DHA
TBHQ	.03	.03	.03	.03	.03	.03	.03	.03

[1] The control diet is Monsanto "US17" diet (Monsanto Co., St. Louis, MO, and Research Diets, Inc., New Brunswick, NJ). Abbreviations: ALA: α-linolenic acid, cLA: conjugated linoleic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, GLA: γ-linolenic acid, SDA: stearidonic acid, TBHQ: tertiary butylhydroquinone.

[2]Trisun is a high-oleic acid sunflower oil (Monsanto, Inc., St. Louis, MO).

[3]Supplied (per kilogram of salt mix): NaCl 259 g, magnesium oxide 41.9 g, $MgSO_4.7H_2O$ 257.6 g, chromium potassium sulfate 1.925 g, cupric carbonate 1.05 g, NaF 0.2 g, KI 0.035 g, ferric citrate 21.0 g, manganous carbonate 12.25 g, ammonium molybdate · 4 H_20 0.3 g, sodium selenite 0.035 g, zinc carbonate 5.6 g, sucrose 399.105 g (Research Diets, Inc., New Brunswick, NJ).

[4]Supplied (per kilogram of vitamin mix): Vitamin A palmitate (500,000 IU per gram) 0.8 g, cholecalciferol (100,000 IU per gram) 1.0 g, vitamin E acetate (500 IU per gram) 10.0 g, menadione sodium bisulfite (62.5% menadione) 0.08 g, biotin (1.0%) 2.0 g, cyanocobalamin (0.1%) 1.0 g, folic acid 0.2 g, nicotinic acid 3.0 g, calcium pantothenate 1.6 g, pyridoxine HCl 0.7 g, riboflavin 0.6 g, thiamin HCl 0.6 g, sucrose, 978.42 g (Research Diets, Inc., New Brunswick, NJ).

TABLE 3-2

Fatty acid composition of the experimental diets[1]

	Dietary groups							
					Omega-3 fatty acids			
	Control	Sulindac	cLA	GLA	ALA	SDA	EPA	DHA
Fatty acid	g/100 g total fatty acids							
12:0	0.36	0.41	0.36	0.37	0.39	0.41	0.42	0.47
14:0	0.56	0.56	0.54	0.55	0.56	0.60	0.60	0.66
16:0	24.91	23.37	23.90	23.93	23.50	25.38	24.28	25.38
16:1	0.15	0.15	ND	0.14	0.14	0.15	0.15	ND
17:0	0.13	0.11	ND	0.12	0.11	0.12	0.12	ND
18:0	13.71	12.38	13.27	12.68	12.24	13.45	12.63	13.45
18:1 n-9	37.56	40.77	26.72	26.28	25.38	27.03	25.85	27.21
18:2 n-6	19.37	18.99	18.89	18.65	18.32	18.72	17.97	18.62
9(Z),11(E) 18:2 n-7	ND	ND	11.05	ND	ND	ND	ND	ND

TABLE 3-2 (continued)

	Control	Sulindac	cLA	GLA	ALA	SDA	EPA	DHA
9(Z),11(Z) 18:2 n-7	ND	ND	2.84	ND	ND	ND	ND	ND
18:3 n-6	ND	ND	ND	14.31	ND	ND	ND	ND
18:3 n-3	1.86	1.83	1.81	1.93	18.38	1.77	1.69	1.74
18:4 n-3	ND	ND	ND	ND	ND	11.31	ND	ND
20:0	0.67	0.60	0.62	0.59	0.56	0.62	0.59	0.57
20:1	0.16	0.16	ND	0.12	0.11	0.11	0.19	ND
20:4 n-6	ND	ND	ND	ND	ND	ND	0.52	ND
20:5 n-3	ND	ND	ND	ND	ND	ND	14.61	ND
22:0	0.38	0.43	ND	0.21	0.20	0.11	0.23	ND
22:6 n-3	0.19	0.18	ND	0.12	0.11	0.23	0.16	11.91
Total saturated FA	40.72	37.86	38.69	38.45	37.56	40.70	38.87	40.52
Total MUFA	37.87	41.15	26.72	26.54	25.64	27.28	26.19	27.21
Total PUFA	21.41	20.99	34.58	35.01	36.80	32.02	34.94	32.27
Total (n-6)	19.37	18.99	18.89	32.96	18.32	18.72	18.48	18.62
Total (n-3)	2.05	2.01	1.81	2.05	18.49	13.30	16.46	13.65

TABLE 3-2 (continued)

	Control	Sulindac	cLA	GLA	ALA	SDA	EPA	DHA
P:S ratio	0.53	0.55	0.89	0.91	0.98	0.79	0.90	0.80
n-3:n-6 ratio	0.11	0.11	0.10	0.06	1.01	0.71	0.89	0.73

[1]The control diet is Monsanto "US17" diet (Monsanto Co., St. Louis, MO, and Research Diets, Inc., New Brunswick, NJ).

Abbreviations: ALA: α-linolenic acid, cLA: conjugated linoleic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, FA: fatty acids, GLA: γ-linolenic acid, MUFA: monounsaturated fatty acids, ND: none detected, P:S ratio: ratio of polyunsaturated fatty acids to saturated fatty acids, PUFA: polyunsaturated fatty acids, SDA: stearidonic acid.

Fatty acid analysis. Fatty acid methyl esters of the tissue phospholipids were prepared and analyzed as described previously (58). Briefly, tissues were homogenized in ice-cold saline and lipids were extracted with chloroform:methanol (1:2 v/v), followed by extraction (x2) with chloroform. The pooled chloroform extracts were evaporated and resuspended in a small volume of chloroform, and the phospholipids were separated by TLC on silica gel 60 HP-TLC plates (Merck, Darmstadt, Germany) with chloroform:methanol (8:1 v/v) as the solvent system. Bands corresponding to the phospholipids were scraped, dissolved in toluene, and saponified with KOH (0.5 mol/L) in methanol for 8 min at 86°C. After acidification with HCl in methanol (0.7 mol/L), the fatty acids were extracted twice with hexane, evaporated, and methylated with ethereal diazomethane. The fatty acid methyl esters were resuspended in hexane and analyzed using a Hewlett-Packard model 5890 series II gas chromatograph equipped with flame ionization detector and a DB23 fused silica capillary column (0.25 mm i.d. x 30 m x 0.25 μm film; J&W Scientific, Folsom, CA). Separation was achieved by temperature programming from 160°C to 250°C at 3.5°C/min with hydrogen as the carrier gas. The internal standard, pentadecaenoic acid (15:0) methyl ester (Avanti Polar Lipids, Alabaster, AL) was added to each sample prior to the saponification step. The fatty acid methyl esters were identified by comparison of retention times with those of known standards (Nu-Chek Prep, Elysian, MN). Various cLA isomers and elongation products of GLA and SDA were identified by comparison of retention times to purified fatty acid methyl ester standards (Matreya, Inc., Pleasant Gap, PA).

Measurement of prostaglandins. After cervical dislocation, normal-appearing intestinal sections were quickly perfused with ice cold saline (NaCl, 154 mmol/L), immediately snap frozen in liquid nitrogen, and stored at -80°C. For analysis, the tissues

were homogenized using a Tenbroeck tissue grinder (Pyrex, England) in ice-cold Tris-HCl buffer (0.1 mol/L, pH 7.4) containing indomethacin (final concentration 1 mmol/L) to inhibit *ex vivo* prostaglandin biosynthesis. This was followed by methanolic acidification with 8.8% formic acid in 90% methanol (final pH 3.5). The resulting homogenate was vigorously mixed and centrifuged, after which the supernatant was removed. The pellet was resuspended in a 20% solution of methanol in water (pH 3.5), vigorously mixed and centrifuged, after which the supernatant was collected and pooled. Prostaglandins were isolated from pooled supernatants by solid-phase extraction using an octadecyl (C18) cartridge (Burdick & Jackson, Muskegon, MI) and eluted with 100% methanol. The recovery of prostaglandins was determined by adding 0.37 nBq [^{3}H]-prostaglandin D_2 (PGD_2) to the sample as an internal standard prior to processing. The methanol was evaporated under a stream of nitrogen gas and the extracts were resuspended in phosphate buffered saline (PBS, pH 7.4) containing gelatin (1.0 g/L). PGE_2 and 6-keto-$PGF_{1\alpha}$ were measured by RIA as described previously using antiserum obtained from PerSeptive Diagnostics, Inc. (Cambridge, MA) (59). Cross-reactivities at half maximal binding of various prostanoids with PGE_2 antiserum are as follows: PGE_2 (100%), PGE_3 (26%), 6-keto-$PGF_{1\alpha}$ (<1%), $PGF_{1\alpha}$ (1%), TXB_2 (<1%), PGD_2 (<1%) and $PGF_{2\alpha}$ (1%). Cross-reactivities at half maximal binding of various prostanoids with 6-keto-$PGF_{1\alpha}$ antiserum are as follows: 6-keto-$PGF_{1\alpha}$ (100%), Δ-17, 6-keto-$PGF_{1\alpha}$ (14%), PGE_2 (<1%), $PGF_{2\alpha}$ (2%), $PGF_{1\alpha}$ (8%), PGD_2 (<1%) and TXB_2 (<1%). All standards were purchased from Cayman Chemical (Ann Arbor, MI); [^{3}H]-PGE_2, [^{3}H]-PGD_2 and [^{3}H]-6-keto-$PGF_{1\alpha}$ were obtained from New England Nuclear (Boston, MA). An aliquot of the homogenate from each sample was used to determine protein concentration by the modified Lowry protocol (60).

Statistical analyses. Values are expressed as means ± SEM. Levene's-test was used to determine homogeneity of variance among groups. Differences in tumor number, tumor size, and biochemical parameters were analyzed statistically by one-way ANOVA followed by Fisher's least significant difference multiple comparison method to determine differences among groups. For statistical analysis of tumor number, one extreme outlier (defined as a value >3 interquartile ranges above the 75^{th} percentile for each of the respective groups) was removed in each of the DHA and sulindac-supplemented groups. This did not affect the statistical results. By the same definition, one extreme outlier was removed from the EPA group for analysis of PGE_2 levels. This altered the statistical significance of PGE_2 levels in the EPA group compared with controls. Regression analysis was used to determine relationships among variables in the fatty acid-supplemented groups. The Statistical Analysis System (SAS Version 6.12, SAS Institute, Inc., Cary, NC) was used to evaluate the data. Repeated measures ANOVA was used to determine differences in body weight gain and dietary intake among groups (SPSS Inc., Chicago, IL). Differences were considered significant at $P < 0.05$.

RESULTS

Food intake and body weights. Repeated measures ANOVA showed no significant difference in weekly weights among the dietary groups throughout the study. Food intake was not significantly different among the groups at each time point by one-way ANOVA. However, repeated measures ANOVA revealed a significant difference in intake over time. This was most likely due to the decline in food intake in the GLA group during the final 2 wk, probably related to the increasing tumor burden toward the end of the study.

Effect of diet treatment on tumor frequency and size. As expected, the animals treated with sulindac (positive control) had 93% fewer overall tumors and significantly smaller tumors relative to the controls **(Table 3-3)**. Supplementation with cLA and GLA had no effect on tumor number or size. Of the n-3 PUFA, dietary SDA and EPA supplementation resulted in the smallest number and size of tumors. ALA, the parent fatty acid in the n-3 family, had no effect, whereas DHA-supplemented mice had 30% fewer tumors (P = 0.15). Interestingly, only SDA-supplemented mice had significantly fewer colonic tumors, which are typically resistant to chemotherapeutic intervention. In this regard, SDA was as effective as sulindac. There were no histologic distinctions between tumors harvested from any of the groups.

Intestinal fatty acid composition. Phospholipid fatty acid composition of normal-appearing small intestinal tissue appropriately reflected dietary fatty acid supplementation **(Table 3-4)**. Conjugated linoleic acid supplementation resulted in detection of cLA isomers in phospholipids at a level of ~2 mol/100 mol. Additionally, 11(Z),13(E)-20:2 n-7, the immediate elongation product of 9(Z),11(E)-18:2 n-7, was detected at low levels (0.02 mol/100 mol). However, we did not detect a conjugated AA derivative of cLA. Feeding cLA had little impact on long-chain PUFA composition of the phospholipids, whereas dietary GLA supplementation significantly enriched phoshpholipids with GLA, DGLA, and AA at the expense of OA and LA. Inclusion of n-3 PUFA in the diets progressively elevated concentrations of EPA and docosapentaenoic acid (DPA, 22:5 n-3) in phospholipids. In contrast, dietary SDA and EPA did not significantly alter phospholipid DHA levels, whereas dietary DHA supplementation more than doubled the DHA content of phospholipids compared with controls. Elevations in levels of n-3 PUFA were primarily at the expense of AA and, in most cases, coincided

TABLE 3-3

Effect of 7 weeks of dietary supplementation with various fatty acid ethyl esters or sulindac on tumor number and size in the colon and small intestine of $Apc^{Min/+}$ mice[1]

	Dietary groups							
					n-3 fatty acids			
	Control (n=10)	Sulindac (n=8)	cLA (n=9)	GLA (n=9)	ALA (n=10)	SDA (n=10)	EPA (n=10)	DHA (n=9)
Colon								
Tumors per group	13	1	10	11	8	2	9	14
Tumors per mouse	1.3±0.6[a]	0.1±0.1[b]	1.1±0.4[ab]	1.2±0.4[a]	0.8±0.3[ab]	0.2±0.1[b]	0.9±0.3[ab]	1.6±0.3[a]
Tumor size[2] (mm)	2.96±0.20[a]	2.00	3.03±0.52[a]	2.57±0.30[a]	2.75±0.46[a]	1.50	2.48±0.39[a]	3.09±0.21[a]
Small intestine								
Tumors per group	337	18	409	421	364	185	176	208
Tumors per mouse	33.7±4.5[ab]	2.3±0.5[d]	45.4±8.2[a]	46.8±7.1[a]	36.4±6.3[ab]	18.5±1.9[c]	17.6±2.2[c]	23.1±3.0[bc]
Tumor size (mm)	1.32±0.05[a]	1.29±0.33[ab]	1.24±0.03[ab]	1.30±0.04[ab]	1.23±0.04[ab]	1.07±0.02[ab]	1.05±0.03[b]	1.13±0.04[ab]

TABLE 3-3 (continued)

Total	Control	Sulindac	cLA	GLA	ALA	SDA	EPA	DHA
Tumors per group	350	19	419	432	372	187	185	222
Tumors per mouse	35.0±4.5^{ab}	2.4±0.5^{d}	46.6±8.3^{a}	48.0±7.4^{a}	37.2±6.6^{ab}	18.7±1.9^{c}	18.5±2.2^{c}	24.7±3.2^{bc}
Tumor size (mm)	1.32±0.04^{ab}	1.04±0.09^{d}	1.28±0.04^{ab}	1.33±0.04^{a}	1.21±0.03^{bc}	1.08±0.02^{d}	1.11±0.04^{cd}	1.21±0.04^{bc}

[1]Values are means ± SEM. Different superscripts within each row indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at $P < 0.05$. The control diet is Monsanto "US17" diet (Monsanto Co., St. Louis, MO, and Research Diets, Inc., New Brunswick, NJ). Abbreviations: ALA: α-linolenic acid, cLA: conjugated linoleic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, GLA: γ-linolenic acid, SDA: stearidonic acid.

[2]Average tumor size was calculated as a weighted average for each size classification and expressed as mean ± SEM.

TABLE 3-4

Fatty acid composition of mouse small intestinal phospholipids after consumption of diets supplemented with various fatty acid ethyl esters or sulindac for 7 wk[1]

	Dietary groups							
					n-3 fatty acids			
	Control (n=10)	Sulindac (n=9)	cLA (n=9)	GLA (n=9)	ALA (n=10)	SDA (n=10)	EPA (n=10)	DHA (n=10)
Fatty acid	*mol/100 mol total fatty acids*							
16:0	20.6±0.3[b]	20.9±1.0[b]	20.0±0.3[b]	20.8±0.6[b]	20.6±0.4[b]	21.5±0.7[ab]	20.6±0.6[b]	22.8±0.2[a]
18:0	21.7±0.3[a]	22.1±0.7[a]	21.6±0.2[ab]	21.7±0.5[a]	22.2±0.5[a]	22.2±0.4[a]	21.9±0.3[a]	20.5±0.3[b]
18:1 n-9	7.2±0.2[b]	8.5±0.4[a]	6.3±0.3[c]	4.8±0.2[e]	6.0±0.1[cd]	6.3±0.2[c]	6.5±0.2[c]	5.6±0.1[d]
18:2 n-6	21.2±0.4[e]	24.5±0.6[c]	22.6±0.7[d]	16.0±0.4[f]	28.2±0.4[b]	29.7±0.4[a]	21.0±0.4[e]	27.3±0.4[b]
9Z, 11E-18:2 n-7	ND[b]	ND[b]	1.6±0.1[a]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
9Z, 11Z-18:2 n-7	ND[b]	ND[b]	0.3±0.1[a]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
9E, 11E-18:2 n-7	ND[b]	ND[b]	<0.1[a]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
18:3 n-6	0.2±0.1[b]	0.2±0.0[b]	0.1±0.0[b]	2.0±0.3[a]	0.2±0.0[b]	0.2±0.0[b]	0.1±0.0[b]	0.1±0.0[b]
18:3 n-3	<0.1[b]	<0.1[b]	<0.1[b]	<0.1[b]	1.1±0.1[a]	<0.1[b]	<0.1[b]	<0.1[b]

TABLE 3-4 (continued)

	Control	Sulindac	cLA	GLA	ALA	SDA	EPA	DHA
18:4 n-3	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	0.3±0.1^{a}	ND^{b}	ND^{b}
11Z,13E-20:2 n-7	ND^{b}	ND^{b}	<0.1^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
20:3 n-6	1.4±0.1^{b}	1.4±0.1^{b}	1.4±0.1^{b}	2.7±0.1^{a}	1.4±0.0^{b}	1.2±0.0^{b}	0.7±0.0^{c}	0.7±0.0^{c}
20:4 n-6	19.8±0.5^{b}	14.2±1.2^{c}	18.4±0.3^{b}	25.3±0.7^{a}	9.2±0.2^{d}	5.9±0.1^{f}	7.6±0.1^{e}	7.0±0.2^{ef}
20:5 n-3	0.3±0.0^{e}	1.0±0.1^{d}	0.4±0.1^{de}	<0.1^{e}	3.2±0.1^{c}	4.7±0.2^{b}	12.9±0.6^{a}	2.8±0.1^{c}
22:4 n-6	0.7±0.0^{b}	0.8±0.2^{ab}	0.7±0.0^{b}	1.2±0.4^{a}	0.1±0.0^{c}	<0.1^{c}	<0.1^{c}	<0.1^{c}
22:5 n-6	0.3±0.0^{c}	0.2±0.0^{d}	0.3±0.0^{c}	0.7±0.0^{b}	ND^{e}	ND^{e}	<0.1^{e}	1.3±0.1^{a}
22:5 n-3	0.3±0.0^{e}	0.5±0.1^{d}	0.3±0.0^{e}	0.2±0.0^{e}	1.0±0.0^{c}	1.5±0.1^{b}	2.8±0.1^{a}	0.3±0.0^{e}
22:6 n-3	5.0±0.2^{cd}	4.4±0.5^{de}	5.0±0.1^{bcd}	3.7±0.2^{f}	5.6±0.2^{b}	5.3±0.2^{bc}	4.3±0.2^{ef}	10.8±0.1^{a}
Total n-3	5.5±0.2^{d}	6.0±0.6^{d}	5.7±0.2^{d}	4.0±0.2^{e}	10.9±0.2^{c}	11.9±0.5^{c}	20.1±0.5^{a}	13.9±0.2^{b}
Total n-6	43.8±0.3^{b}	41.4±1.5^{c}	45.4±0.5^{b}	48.0±0.5^{a}	39.2±0.5^{d}	37.1±0.4^{e}	29.6±0.4^{f}	36.4±0.4^{e}
n-3/n-6 ratio	0.1±0.0^{e}	0.1±0.0^{e}	0.1±0.0^{e}	0.1±0.0^{f}	0.3±0.0^{d}	0.3±0.0^{c}	0.7±0.0^{a}	0.4±0.0^{b}

[1]Values are means ± SEM. Different superscripts within each row indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at $P < 0.05$. Abbreviations: ALA: alpha-linolenic acid, cLA: conjugated linoleic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, GLA: γ-linolenic acid, ND: none detected, SDA: stearidonic acid.

with increases in LA content. SDA, the Δ-6 desaturase product of ALA, was the most effective fatty acid in reducing AA content, and with the exception of dietary EPA, was the fatty acid associated with the lowest AA content, and with the exception of dietary EPA, was associated with the highest tissue EPA content. Only minimal amounts of SDA were detected in intestinal phospholipids.

Prostaglandin levels in normal-appearing small intestine. GLA and cLA did not significantly alter basal prostaglandin levels (PGE_2 and 6-keto-$PGF_{1\alpha}$) in normal-appearing small intestinal tissue **(Table 3-5)**. In contrast, dietary n-3 PUFA supplementation resulted in basal prostaglandin levels significantly lower than controls by ~50%, which coincided with similarly low levels of tissue AA. SDA and EPA were the dietary fatty acids associated with the lowest prostaglandin levels. Furthermore, treatment with sulindac, an inhibitor of cyclooxygenase, the committed step in prostaglandin biosynthesis, resulted in prostaglandin levels 56% lower than those observed in control mice.

DISCUSSION

This study clearly establishes the anti-tumorigenic properties of dietary n-3 fatty acids in *Apc*$^{Min/+}$ mice. We previously reported that dietary EPA reduced tumor load by 50% and that this effect was due, in part, to antagonism of AA metabolism (6). The current study expands on these earlier results by investigating the anti-tumorigenic effects of EPA and other n-3 PUFA, *viz.* ALA, SDA and DHA, within the context of a typical Western diet. As observed previously, EPA-supplemented mice had ~50% fewer tumors, confirming EPA's anti-tumorigenic capacity in this animal model. Similarly, SDA-supplemented mice had an equally low number of intestinal tumors. In addition, it

TABLE 3-5

Basal prostaglandin levels in normal-appearing small intestines of mice after consumption of diets supplemented with various fatty acid ethyl esters or sulindac for 7 wk[1]

	Dietary groups							
					n-3 fatty acids			
	Control	Sulindac	cLA	GLA	ALA	SDA	EPA	DHA
	(n=10)	(n=9)	(n=9)	(n=9)	(n=10)	(n=10)	(n=9-10)[2]	(n=10)
Prostaglandin	*ng/mg protein*							
PGE_2	37.4±8.9[a]	16.7±4.4[b]	31.5±6.9[ab]	30.7±4.8[ab]	20.3±4.9[b]	17.8±3.1[b]	16.6±2.5[b]	19.9±3.0[b]
6-keto-$PGF_{1\alpha}$	10.1±1.5[a]	4.4±0.9[c]	9.7±1.2[ab]	8.3±0.7[ab]	6.0±0.7[bc]	5.2±0.7[c]	4.6±0.4[c]	5.5±0.7[c]

[1]Values are means ± SEM. Different superscripts within each row indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at $P < 0.05$. Abbreviations: ALA: alpha-linolenic acid, CLA: conjugated linoleic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, GLA: γ-linolenic acid, 6-keto-$PGF_{1\alpha}$: 6-keto-Prostaglandin $F_{1\alpha}$, PGE_2: Prostaglandin E_2, SDA: stearidonic acid.

[2]See Materials and Methods for explanation.

was the only dietary fatty acid associated with significantly fewer tumors in the colon. Tumor number and size were not lower, however, in ALA-supplemented mice. This is in contrast to previous studies which demonstrated a protective effect of perilla oil (as the source of ALA), on chemically-induced colorectal tumors in a rat model (18, 19). This difference may be due to distinct mechanisms of tumorigenesis and/or contrasting dietary designs. In both of these studies, perilla oil replaced safflower oil, which is rich in the tumor-promoting PUFA LA (15). On the other hand, we designed our diets to control for variations in fatty acid content within the context of a typical Western diet, mimicking relative distributions of macronutrient and fatty acid composition. Our experimental design used OA as the control fatty acid, thus maintaining constant levels of n-6 fatty acids in our diets. We previously showed OA to be neutral with respect to prostaglandin biosynthesis and tumorigenesis in *Apc*$^{Min/+}$ mice (unpublished results). This unique dietary design allows us to avoid problems associated with substituting one dietary oil for another in that any biological or biochemical effects observed can only be attributed to the individual fatty acids supplemented to the diets.

ALA is the parent fatty acid of the n-3 family of fatty acids and is metabolized to SDA via Δ-6 desaturase, the rate-limiting step in this biosynthetic pathway (26). Our results suggest that desaturation of ALA to SDA via the Δ-6 desaturase may be an important step involved in the anti-tumorigenic effect of n-3 fatty acids. However, it is unclear whether these differences are related to the formation of down-stream metabolites, the ability to antagonize AA and its metabolism (highly unsaturated n-3 PUFA are 2.5 to 5 times more effective than ALA) (61), and/or whether the degree of unsaturation of the n-3 fatty acids has an independent effect on cell signaling/function. Feeding SDA did not dramatically enrich tissue phospholipids with SDA; its ability to alter

intestinal tumor load appears to be related more to its conversion to EPA than DHA given the fact that SDA had no effect on DHA levels and DHA-supplemented mice appeared to have only modestly fewer tumors. With the exception of EPA, dietary SDA had the greatest effect on elevating tissue EPA content, but was the most potent fatty acid in its ability to lower tissue AA levels. Similarly, supplementing rats with dietary SDA (1 g/100 g) resulted in significantly elevated levels of EPA, but not SDA, in hepatic phospholipid, triglyceride and free fatty acid fractions, whereas phospholipid AA levels were significantly lower compared with controls (25). Rescuing tissue AA levels by supplementing AA to diets containing EPA effectively normalizes tumor load in $Apc^{Min/+}$ mice, highlighting the importance of AA in maintaining tumor integrity (6).

The ability of dietary DHA to reduce intestinal tumorigenesis effectively is more equivocal than that of SDA or EPA. DHA-supplemented mice appeared to have fewer tumors, but these differences were not significant ($P = 0.15$). However, partial retroconversion of DHA to EPA and its subsequent impact on AA may have accounted for its less-pronounced efficacy compared with EPA itself. Although we cannot rule out any independent effects of DHA, there was no significant correlation between DHA levels in the tissue phospholipids and tumor number. In the only other study examining the efficacy of dietary DHA in a similar animal model, DHA resulted in fewer tumors only in female $Apc^{\Delta 716}$ mice, but not in their male counterparts (16). Overall, these data suggest that the ability of SDA and EPA to lessen tumor multiplicity is not dependent upon their conversion to DHA.

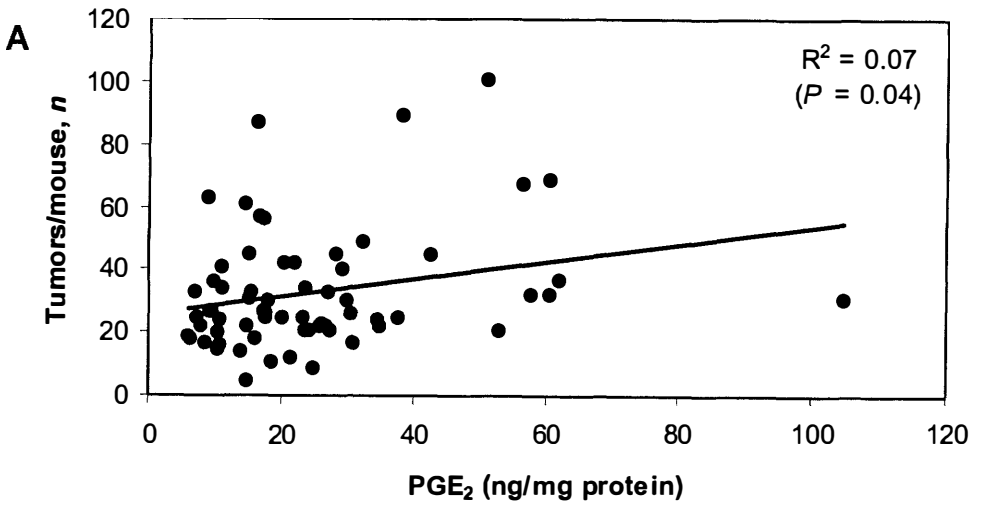
The ability of dietary fatty acids to modify prostaglandin levels most likely contributes to their influence on tumorigenesis. Prostaglandin involvement in intestinal tumorigenesis was recently demonstrated in a study using prostaglandin E receptor (EP)

knockout mice and an EP receptor antagonist (62). Currently, four EP receptors have been identified (EP1-EP4). Watanabe et al. (1999) treated C57BL/6J mice with the colon carcinogen azoxymethane (AOM) and observed significantly fewer early neoplastic lesions (ACF) in EP1 receptor knockout mice compared with wild-type controls. Similarly, AOM-treated wild-type mice dose-dependently developed fewer ACF after administration of ONO-8711, an EP1 receptor antagonist. They also showed that ONO-8711 resulted in 44% fewer tumors in $Apc^{Min/+}$ mice (62), confirming the importance of EP1 and PGE to *Apc*-mediated tumorigenesis. The EP1 receptor acts through a phospholipase C-mediated signaling pathway resulting in the potential activation of protein kinase C following the release of diacylglycerol. Over-expression of protein kinase C β_{II} results in downregulation of glycogen synthase kinase 3β (GSK-3β), an elevation in cellular β-catenin levels, and proliferation of colonic epithelium (63). The *Apc* gene product acts in concert with GSK-3β to regulate the wnt/β-catenin signaling pathway (64). Loss of full-length *Apc* protein, as occurs in the $Apc^{Min/+}$ mouse model, disables the cell's ability to down-regulate β-catenin and, as a result, free (not bound to E-cadherin) β-catenin increases in the cytoplasm and moves into the nucleus where it acts as a co-transcription factor with LEF/TCF to induce expression of target genes (64). Treatment with NSAIDs, inhibitors of prostaglandin biosynthesis, may reduce tumor loads in this model, at least in part, by attenuating β-catenin levels (65, 66) and n-3 PUFA may also modulate this signaling pathway via reductions in AA and PKC activation (67).

In our study, prostaglandin levels were significantly lower in all n-3 fatty acid supplemented groups, with dietary EPA having the greatest impact. However, lower prostaglandin levels did not always equate to a lower tumor number. For example, ALA significantly attenuated prostaglandin levels, but did not alter tumor multiplicity,

suggesting that prostaglandins play a partial role in tumorigenesis. Furthermore, regression analysis revealed a small, but significant relationship between prostaglandin levels and tumor number, with prostaglandins accounting for 7-17% of the variability in tumor number (**Fig. 3-1A**, **3-1B**). It is possible that the degree of unsaturation associated with the n-3 fatty acids could differentially affect the expression or, more importantly, the activity of COX (68), the committed step in prostaglandin biosynthesis (69, 70). Intestinal tumors in *Apc*$^{Min/+}$ mice express both isozymes of COX: COX-1, which is constitutively expressed, and COX-2, the inducible isoform (71-73). Dietary fish oils (rich in EPA and DHA) reduce COX-2 expression in an AOM-induced rat colonic tumor model (70) and immunoreactive COX-1 and -2 protein levels in DMBA-induced mammary tumors (69), whereas n-6 fatty acids increase COX-2 expression (70). The involvement of COX in this animal model is clearly established: 1) inhibition of the COX isozymes by non-selective inhibitors results in 85-96% fewer tumors (51, 74-76), 2) selective inhibition of COX-2 results in 52% fewer tumors (77), 3) crossing COX-2 knockout mice with *Apc*$^{\Delta 716}$ knockout mice results in fewer tumors in a gene-dose responsive manner (78), and 4) crossing COX-1 knockout mice with *Apc*$^{Min/+}$ mice results in 80-90% fewer tumors (79). Nevertheless, the precise mechanisms underlying the anti-tumorigenic effects of SDA and EPA remain unclear.

Dietary supplementation of cLA and GLA did not alter tumorigenesis in this study. This is of particular interest because cLA has been shown to be a potent anti-tumorigenic agent in chemically-induced mammary tumors in rats at levels $\leq$1 g/100 g diet regardless of the level or type of dietary fat (31, 80). It has recently been suggested that cLA may exert its anti-tumorigenic effect by inhibiting the conversion of LA to AA and, ultimately, to eicosanoids (32-34). Banni et al. (1999) showed that cLA at a level of 1 g/100 g diet



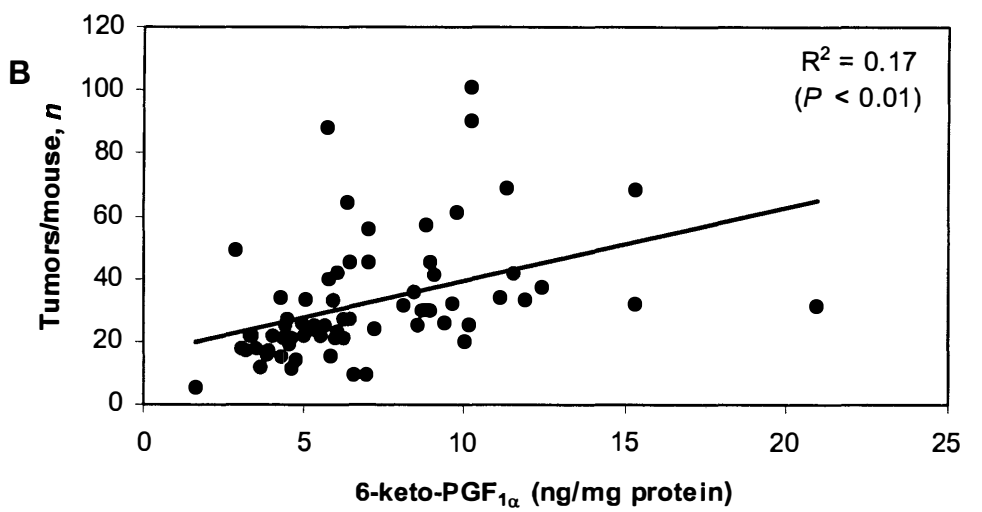


FIGURE 3-1. The relationship between total tumor numbers of male *Apc*$^{Min/+}$ mice fed various fatty acid ethyl esters for 7 wk and prostaglandin levels in samples of normal-appearing small intestinal tissue. Regression analysis showed a significant correlation between PGE_2 and tumor number (Fig. 1A) and 6-keto-$PGF_{1\alpha}$ and tumor number (Fig. 1B) as indicated in the upper right-hand corner of each figure.

maximally lowered GLA, DGLA, and AA levels as a percentage of total lipid in normal mammary tissue (32). Others have detected conjugated AA derivatives of cLA in hepatic lipids of rats after intragastric cLA administration (81). However, we did not observe any alterations in the small intestinal phospholipid content of LA metabolites or detect conjugated AA and, concomitantly, we did not observe any reductions in differences in tumor number or size. Earlier evidence suggested that cLA has anti-tumorigenic potential in the gastrointestinal tract by reducing chemically-induced colonic ACF in rats and gastric neoplasia in mice after oral administration by gavage (35, 36). In contrast, others reported that dietary cLA had no effect on carcinogen-DNA adduct formation in colons of rats treated with 2-amino-3-methylimidazo(4,5-*f*)quinoline or 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (82). The effectiveness of cLA on intestinal tumorigenesis could be related to the type of feeding regimen or the underlying mechanisms responsible for tumor initiation and progression. Nonetheless, our study is the first to investigate the effectiveness of dietary cLA on intestinal tumorigenesis in $Apc^{Min/+}$ mice and we observed no effect on tumor number or size.

To date, the efficacy of dietary GLA on intestinal tumorigenesis has not been determined. Therapeutic studies involving dietary GLA have largely utilized the DMBA-induced rat mammary tumor model or nude mouse mammary carcinoma xenograft model with encouraging results (48, 49). The use of GLA in the current study, however, did not alter intestinal tumor load in $Apc^{Min/+}$ mice, although tissue DGLA levels were significantly higher in the phospholipid fraction of the intestines. DGLA can be metabolized to 15-HETrE and PGE_1. Both of these compounds reportedly have anti-proliferative properties (as reviewed by Fan & Chapkin, 1998) (83). Like PGE_2, the actions of PGE_1 are mediated via the G-protein coupled EP receptors (83-85). However,

recent evidence indicates that antagonism, not agonism, of the EP1 receptor results in significant reductions in tumor load in this mouse model (62). Furthermore, we found that feeding GLA resulted in 28% higher AA concentrations in intestinal phospholipids, although levels of PGE_2 and 6-keto-$PGF_{1\alpha}$ were not significantly different from control values, possibly accounting for the lack of efficacy of GLA. We determined previously that elevating tissue AA levels and prostaglandin levels, for that matter, above control values had no effect on tumor number or size in this animal model (6, 51).

In summary, SDA and EPA supplementation resulted in ~50% fewer intestinal tumors in *Apc*$^{Min/+}$ mice, a genetic murine model of colorectal cancer based on a mutation of the *Apc* tumor suppressor gene. SDA was the most efficacious n-3 fatty acid, resulting in significantly fewer tumors in both the colon and small intestine plus significantly smaller tumors in the small intestine. The anti-tumorigenic effects of both SDA and EPA are likely related to alterations in tissue AA content and prostaglandin levels (6). The fact that dietary ALA had no effect on tumor multiplicity suggests the importance of its conversion to SDA via the Δ-6 desaturase, whereas the ability of DHA to affect tumorigenesis is less clear. Like ALA, cLA and GLA had no effect on intestinal tumorigenesis in this model.

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PART IV

Antagonism of Arachidonic Acid is Linked to the Anti-Tumorigenic Effect of Dietary Eicosapentaenoic Acid in *Apc*$^{Min/+}$ Mice

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ABSTRACT

The multiple intestinal neoplasia (*Apc*$^{Min/+}$) mouse possesses a germline mutation at codon 850 of the adenomatous polyposis coli (*Apc*) gene resulting in the formation of a non-functional truncated gene product. Following a somatic mutation of the remaining wild type allele, mice spontaneously develop approximately 40 to 50 tumors throughout the intestinal tract. This mouse model has been used to study intestinal tumorigenesis because this mutation is analogous to the inherited *APC* mutation in humans with familial adenomatous polyposis (FAP). These individuals characteristically develop numerous adenomas throughout their intestinal tracts. Only a few studies have evaluated the effects of dietary fatty acids on tumorigenesis in this animal model with varying results and none have linked these effects to alterations in arachidonic acid (AA) metabolism. This study was designed to evaluate the anti-tumorigenic effect of dietary (n-3) polyunsaturated fatty acids in the *Apc*$^{Min/+}$ mouse model and to determine if these effects are related to inhibition of AA metabolism. Male *Apc*$^{Min/+}$mice were fed diets supplemented with EPA, AA or a combination of AA+EPA. Mean tumor number in the EPA group was 68% lower ($P < 0.05$) as compared to the control group, while AA supplementation did not significantly alter tumor load. The reduction in tumor load coincided with significant reductions in intestinal AA content and levels of prostaglandins. However, supplementing AA to the EPA diet (AA+EPA) abolished the anti-tumorigenic effect of EPA, increased tissue AA content 4-fold and prostaglandin production 3-5 fold. These results indicate that AA is involved in tumorigenesis and suggest that EPA's ability to reduce tumor load in *Apc*$^{Min/+}$ mice is related to reductions in tissue AA content or its metabolism.

INTRODUCTION

Colorectal cancer persists as the second leading cause of cancer deaths in the United States with a reported 56,503 deaths in 1997 (1). Epidemiological studies indicate that consumption of fish oil correlates with a reduced risk of colorectal cancer (2, 3). Fish oil is rich in n-3 polyunsaturated fatty acids (PUFA), *viz.,* eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Studies in humans and in chemically-induced colonic tumor animal models overwhelmingly indicate a protective effect of n-3 PUFA and the mechanism is largely thought to be related to a reduction in prostaglandin biosynthesis (4-14).

Studies of the anti-tumorigenicity of n-3 PUFA in the $Apc^{Min/+}$ mouse model have been more equivocal. This is the first study to report that purified EPA reduces intestinal tumors in this mouse model and that the anti-tumorigenicity is related to arachidonic acid (AA, 20:4 n-6) metabolism. Over the last several years, the $Apc^{Min/+}$ mouse model has been used to evaluate the effects of nutrition intervention on intestinal tumorigenesis because of its germline mutation in the murine *Apc* gene (15-17). Development of colorectal cancer in humans from dysplastic crypts to metastatic carcinoma involves a series of genetic mutations, the earliest often involving *APC* (18, 19). Individuals with familial adenomatous polyposis (FAP) carry a germline mutation in *APC* and mutational damage or loss of the wild type allele initiates intestinal tumor formation (20, 21). Somatic mutations resulting in loss of full length *APC* protein also occur early in spontaneous forms of the disease (18) indicating an *APC* defect is associated with a majority of human colorectal cancers (22, 23).

$Apc^{Min/+}$ mice spontaneously develop adenomas throughout the intestinal tract with preferred localization in the small intestines (24). These adenomas have been

shown to be sensitive to modulators of the arachidonic acid (AA, 20:4 n-6) pathway. A number of nonsteroidal anti-inflammatory drugs (NSAIDs) can significantly reduce tumor load in this model (24-27) and these effects have been attributed to inhibition of prostaglandin biosynthesis (11, 13, 14, 25). Similarly, dietary n-3 PUFA can reduce tumor load in mice with an *Apc* gene defect. Paulsen et al. (1997) found that a fish oil concentrate enriched with EPA (54%) and DHA (30%) reduced tumor number and size in $Apc^{Min/+}$ mice with more consistent effects in female mice as compared to their male counterparts (16). In comparison, Oshima et al. (1995), using $Apc^{\Delta 716}$ knockout mice, showed that dietary DHA decreased tumor number in female, but not male mice (15). However, antagonism of AA or its metabolism has not yet been established as the driving force behind the anti-tumorigenicity of n-3 PUFA.

We have previously shown that dietary EPA potently antagonized incorporation of AA into phospholipids while increasing tissue EPA and inhibiting prostaglandin formation (28). Conversely, feeding AA systemically increased tissue AA content and eicosanoid formation *in vivo* and supplementing AA to a diet containing EPA completely eliminated the effects of dietary EPA (28). Therefore, in the present study, we hypothesized that feeding EPA would reduce tumor load in male $Apc^{Min/+}$ mice by antagonizing AA metabolism, and that providing AA and EPA in the diet concomitantly would eliminate this effect (i.e., rescue the tumors), suggesting the beneficial effects of n-3 PUFA are mediated through AA.

MATERIALS AND METHODS

Animals. Twenty male C57BL/6J $Apc^{Min/+}$ mice (Jackson Laboratories, Bar Harbor, ME), 38-43 days of age, were randomized into four dietary groups (n = 5

mice/group) on arrival. They were housed in a temperature-controlled room with 12 h periods of light and dark and given free access to food and water. The health of the animals was checked daily. Food was withheld from them overnight before being killed. All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Diets. All diets were based on the composition of the AIN-93G diet and composed of fat-free powder diet (900 g/kg) (Dyets, Inc., Bethlehem, PA) plus soybean oil (70 g/kg). In addition, the diet was supplemented with 30 g/kg purified fatty acid ethyl esters in the form of oleic acid (OA, 18:1 n-9) (30 g/kg), AA (15 g/kg) + OA (15 g/kg), EPA (15 g/kg) + OA (15 g/kg), or AA (15 g/kg) + EPA (15 g/kg) **(Table 4-1)**. Overall fatty acid composition of the diet is shown in **Table 4-2**. The ethyl esters of OA and AA were purchased from Nu-Chek Prep (Elysian, MN) and the EPA ethyl ester (> 97% purity) was kindly supplied by the National Oceanographic and Atmospheric Administration (Charleston, SC). The ethyl ester of OA was used as a control because we have previously demonstrated that supplementing OA in the diet at the levels used in this study has no effect on tissue contents of AA and EPA and eicosanoid biosynthesis (unpublished results). All diets were supplemented with the antioxidant tertiary butylhydroquinone (Eastman Chemical Company, Kingsport, TN) at a level of 20mg/kg diet to limit oxidation of the PUFA added to the diets. All diets were prepared in the absence of UV light, immediately divided into daily aliquots and stored under an atmosphere of nitrogen at -80°C to prevent oxidation. All animals were provided with fresh food daily. Animal weights were recorded weekly.

TABLE 4-1

Experimental diet composition

Component[1]	Dietary Groups			
	OA[2]	AA	EPA	AA+EPA
		g/kg		
Fat Free Diet[3]	900	900	900	900
Soybean Oil	70	70	70	70
18:1 n-9 ethyl ester	30	15	15	--
20:4 n-6 ethyl ester	--	15	--	15
20:5 n-3 ethyl ester	--	--	15	15
TBHQ[4]	20 mg	20 mg	20 mg	20 mg

[1]All ingredient values are expressed as g/kg diet except TBHQ.

[2]Abbreviations: OA: oleic acid (control), AA: arachidonic acid, EPA: eicosapentaenoic acid.

[3]Fat-free purified rodent diet (AIN-93G, DYETS, Inc. Bethlehem, PA). Composition (g/kg final diet mix): casein, 196.7; cornstarch, 379.9; DYETROSE, 126.1; sucrose, 98.4; cellulose, 49.2; AIN-93G-MX mineral mixture, 34.4; AIN-93-VX vitamin mixture, 9.8; L-cystine, 3.0; choline bitartrate, 2.5 (American Institute of Nutrition 1993).

[4]TBHQ: tertiary butylhydroquinone.

TABLE 4-2

Fatty acid composition of the experimental diets[1]

	Dietary Groups			
	OA	AA	EPA	AA+EPA
	g/100 g total fatty acids[2]			
Fatty acid				
16:0	7.4	7.5	7.6	7.7
18:0	3.6	3.6	3.7	3.7
18:1 n-9	46.6	32.0	32.4	17.4
18:2 n-6	35.9	37.0	36.5	37.5
18:3 n-3	4.7	4.8	4.9	4.9
20:4 n-6	ND	13.3	ND	13.5
20:5 n-3	ND	<0.08	13.4	13.7

[1]Abbreviations: OA: oleic acid, AA: arachidonic acid, EPA: eicosapentaenoic acid, ND: none detected.

[2]Individual percentages do not total 100% as fatty acids present at levels below 0.5% are not shown.

Experimental design. Upon arrival, all animals were initially provided a chow diet (Harlan Teklad, Madison, WI) prior to the start of the experimental diets at 42-45 days of age. Animals were maintained on assigned diets for approximately 8 weeks. At 98-100 days of age, they were terminated by cervical dislocation and tumor number, size, and location were determined as previously described (24). Portions of normal-appearing intestine and several of the largest tumors were harvested for histological examination. Basal levels and *ex vivo* production of prostaglandin E_2 (PGE_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) were determined for each animal from samples of normal-appearing small intestine.

Fatty acid analysis. The fatty acid methyl esters of the tissue phospholipids were prepared and analyzed as described previously (29). Briefly, tissues were homogenized in ice-cold saline and lipids were extracted with chloroform:methanol (1:2 v/v), followed by extraction (x2) with chloroform. The pooled chloroform extracts were evaporated and resuspended in a small volume of chloroform, and the phospholipids were separated by TLC on silica gel 60 HP-TLC plates (Merck, Darmstadt, Germany) with chloroform:methanol (8:1 v/v) as the solvent system. Bands corresponding to the phospholipids were scraped, dissolved in toluene, and saponified with KOH 0.5 mol/L in methanol for 8 minutes at 86°C. After acidification with HCl 0.7 mol/L in methanol, the fatty acids were extracted with hexane (x2), evaporated, and methylated with ethereal diazomethane. The fatty acid methyl esters were resuspended in hexane and analyzed using a Hewlett-Packard model 5890 series II gas chromatograph equipped with flame ionization detector and a DB23 fused silica capillary column (0.25 mm i.d. x 30 m x 0.25 µm film; J&W Scientific, Folsom, CA). Separation was achieved by temperature programming from 160°C to 250°C at 3.5°C/minute with hydrogen as the carrier gas.

The internal standard, pentadecaenoic acid (15:0) methyl ester (Avanti Polar Lipids, Alabaster, AL) was added to each sample prior to the saponification step. The fatty acid methyl esters were identified by comparison of retention times with those of known standards (Nu-Chek Prep, Elysian, MN).

Measurement of prostaglandins. Following cervical dislocation, normal-appearing intestinal sections were quickly perfused with ice cold saline (NaCl, 154 mmol/L), immediately snap frozen in liquid nitrogen, and stored at -80°C. For analysis, the tissues were homogenized using a Polytron homogenizer in ice-cold Tris-HCl buffer (0.1 mol/L, pH 7.4). Prostaglandin production was determined by incubating the homogenate for 0 minutes (basal) or 15 minutes (*ex vivo*) at 37°C. Indomethacin (final conc. 1 mmol/L) was added followed by formic acid in methanol (pH 3.0) to arrest prostaglandin synthesis. Prostaglandins were isolated by solid-phase extraction using an octadecyl (C18) cartridge (Burdick & Jackson, Muskegon, MI) and eluted with 100% methanol. The recovery of prostaglandins was determined by adding 0.37 nBq [^{3}H]-Prostaglandin D_2 (PGD_2) to the sample as an internal standard prior to processing. The methanol was evaporated under a stream of nitrogen gas and the extracts were resuspended in phosphate buffered saline (PBS, pH 7.4) containing gelatin (1.0 g/L). PGE_2 and 6-keto-$PGF_{1\alpha}$ were measured by RIA as described previously using antiserum obtained from PerSeptive Diagnostics, Inc. (Cambridge, MA) (29). Cross-reactivities at half maximal binding of various prostanoids with PGE_2 antiserum are as follows: PGE_2 (100%), PGE_3 (26%), 6-keto-$PGF_{1\alpha}$ (<1%), $PGF_{1\alpha}$ (1%), TXB_2 (<1%), PGD_2 (<1%) and $PGF_{2\alpha}$ (1%). Cross-reactivities with various prostanoids with 6-keto-$PGF_{1\alpha}$ antiserum are as follows: 6-keto-$PGF_{1\alpha}$ (100%), Δ-17, 6-keto-$PGF_{1\alpha}$ (14%), PGE_2 (<1%), $PGF_{2\alpha}$ (2%), $PGF_{1\alpha}$ (8%), PGD_2 (<1%) and TXB_2 (<1%). All standards were

purchased from Cayman Chemical (Ann Arbor, MI), and [^{3}H]-PGE_2, [^{3}H]-PGD_2 and [^{3}H]-6-keto-$PGF_{1\alpha}$ were obtained from New England Nuclear (Boston, MA). An aliquot of the homogenate from each sample was used to determine protein concentration by the modified Lowry protocol (30).

Statistical analyses. Values are expressed as means ± SEM. Differences in final body weights, tumor number, tumor size, and biochemical parameters were analyzed statistically by one-way ANOVA followed by Fisher's least significant difference multiple comparison method to determine differences among groups. The Statistical Analysis System (SAS Version 6.12, SAS Institute, Inc., Cary, NC) was used to evaluate the data. Square root transformations of raw data were performed in cases of unequal variances. Differences were considered significant at $P < 0.05$.

RESULTS

Body weights. At the end of the study, the average weight of the animals in the OA group was significantly lower than those of EPA and AA+EPA groups, most likely a reflection of the average tumor load in the respective groups **(Fig. 4-1)**. The only animals still gaining weight at the end of the study were in the EPA group. It has been our experience that increasing tumor load has a marked negative impact on growth patterns in these mice after 75 days of age (24).

Intestinal fatty acid composition. Including AA, EPA, or AA+EPA in the diets resulted in characteristic changes in phospholipid fatty acid composition (28, 29) **(Table 4-3)**. Tissue AA (20:4 n-6) levels from the AA group were significantly higher compared to the OA group (control), while the levels of linoleic acid (18:2 n-6) were significantly lower. Including EPA (20:5 n-3) in the diet enriched phospholipids with EPA, with a

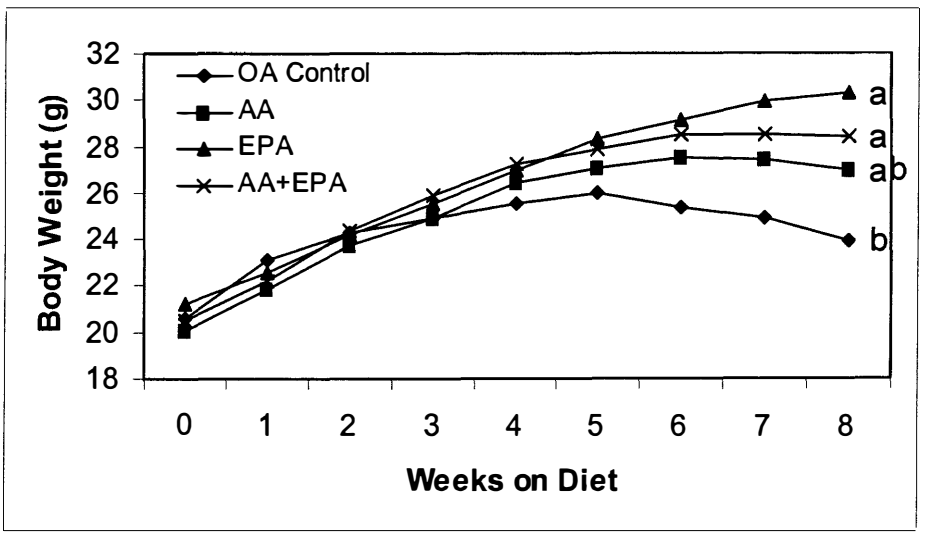

FIGURE 4-1. Weekly weights of male *Apc*$^{Min/+}$ mice fed diets supplemented with oleic acid (OA, control), arachidonic acid (AA), eicosapentaenoic acid (EPA), or eicosapentaenoic acid plus arachidonic acid (AA+EPA) at a level of 15 g/kg diet for eight wk. Data are means ± SEM, n = 5 in each group. Final weights were evaluated for statistical significance by one-way ANOVA and differences among groups were determined by Fisher's least significant difference multiple comparison method. Different superscripts indicate a significant difference at $P < 0.05$.

TABLE 4-3

Fatty acid composition of mouse small intestine phospholipids after consumption of diets supplemented with fatty acid ethyl esters for 8 wk[1]

	Dietary groups			
	OA (n=5)	AA (n=5)	EPA (n=5)	AA + EPA (n=5)
Fatty acid	*mol/100 mol*[2]			
16:0	19.38 ± 0.21[b]	19.47 ± 0.70[b]	18.92 ± 0.58[b]	22.44 ± 0.39[a]
16:1	0.14 ± 0.06[b]	0.27 ± 0.04[b]	0.47 ± 0.08[a]	0.19 ± 0.05[b]
18:0	21.74 ± 0.43[b]	23.48 ± 0.47[a]	23.04 ± 0.27[a]	23.06 ± 0.35[a]
18:1 n-9	7.03 ± 0.21[a]	4.62 ± 0.32[c]	6.11 ± 0.28[b]	3.19 ± 0.06[d]
18:2 n-6	23.71 ± 0.67[b]	15.32 ± 0.44[c]	26.73 ± 1.13[a]	17.33 ± 0.30[c]
20:3 n-6	0.90 ± 0.05[b]	0.40 ± 0.01[c]	1.16 ± 0.05[a]	0.39 ± 0.02[c]
20:4 n-6	16.65 ± 0.19[c]	28.24 ± 0.74[a]	6.21 ± 0.34[d]	25.09 ± 0.72[b]
20:5 n-3	0.28 ± 0.03[c]	0.19 ± 0.06[c]	7.09 ± 0.41[a]	1.03 ± 0.04[b]

TABLE 4-3 (continued)

	OA	AA	EPA	AA + EPA
22:4 n-6	1.39 ± 0.43[a]	1.41 ± 0.07[a]	0.10 ± 0.03[b]	0.64 ± 0.05[b]
22:5 n-6	0.54 ± 0.18[a]	0.54 ± 0.06[a]	0.03 ± 0.01[b]	0.02 ± 0.02[b]
22:5 n-3	0.37 ± 0.02[c]	0.41 ± 0.02[c]	2.78 ± 0.30[a]	1.34 ± 0.11[b]
22:6 n-3	4.74 ± 0.11[a]	3.51 ± 0.35[b]	4.77 ± 0.37[a]	3.31 ± 0.29[b]
Total n-3	5.47 ± 0.17[b]	4.19 ± 0.33[b]	14.81 ± 0.94[a]	5.79 ± 0.39[b]
Total n-6	43.65 ± 0.93[b]	46.34 ± 0.41[a]	34.71 ± 1.28[c]	43.85 ± 0.61[ab]
n-3/n-6 ratio	0.13 ± 0.00[b]	0.09 ± 0.01[b]	0.43 ± 0.04[a]	0.13 ± 0.01[b]

[1]Values are means ± SEM. Different superscripts within each row indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at $P < 0.05$.

[2]Abbreviations: OA: oleic acid (control), AA: arachidonic acid, EPA: eicosapentaenoic acid.

concomitant decline in tissue AA levels. When AA and EPA were simultaneously included in the diet (AA+EPA), tissue AA levels were 4-fold higher and tissue EPA levels were lower (by 85%) compared to the EPA group.

Effect of diet treatment on tumor frequency, size, and morphology. Tumor number was not significantly different in the AA-supplemented group as compared to the OA (control) group; however, animals in the EPA group had 68% and 54% fewer tumors than animals in the OA and AA groups, respectively ($P < 0.05$) **(Table 4-4)**. When AA was supplemented to the EPA-containing diet (AA+EPA), the average number of tumors was more than twice that observed in the EPA group, duplicating the results of the AA group. The tumors, on average, were significantly smaller in the EPA group as compared to the other dietary groups and supplementation of AA to the EPA-fed animals resulted in significantly larger tumors ($P < 0.05$). Overall, more than 98% of tumors were located in the small intestines.

Prostaglandin production by normal-appearing small intestine. *Ex vivo* prostaglandin production was significantly correlated with tissue AA content as shown in **Table 4-5**. Basal levels of PGE_2 and 6-keto-$PGF_{1\alpha}$ were consistently lower in the EPA-fed group compared to the OA and AA groups **(Table 4-6)** and including AA in the diet that contained EPA (AA+EPA) resulted in PGE_2 and 6-keto-$PGF_{1\alpha}$ levels that were significantly higher compared to the group supplemented with EPA alone. *Ex vivo* prostaglandin production was also significantly higher in the diet containing AA compared to the OA and EPA groups, while the EPA group had the lowest production compared to all other groups. The addition of AA to the EPA group (AA+EPA) eliminated any reductions in prostaglandin production due to EPA supplementation, with the exception of basal 6-keto-$PGF_{1\alpha}$, as compared to the OA control group.

TABLE 4-4

Effect of EPA and AA+EPA on tumor number and size in the colon and small intestine of $Apc^{Min/+}$ mice[1]

	Dietary Groups			
	OA[2] (n=5)	AA (n=5)	EPA (n=5)	AA+EPA (n=5)
Total tumors by group				
Colon	4	2	4	4
Small intestine	334	237	106	237
Total tumors/group	338	239	110	241
Tumors/mouse	68 ± 9^{a}	48 ± 9^{a}	22 ± 4^{b}	48 ± 6^{a}
Tumor size (mm)	1.35 ± 0.02^{a}	1.38 ± 0.05^{a}	1.11 ± 0.07^{b}	1.33 ± 0.04^{a}

[1]Values are means ± SEM. Different superscripts within each row indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at $P < 0.05$.

[2] Abbreviations: OA: oleic acid (control), AA: arachidonic acid, EPA: eicosapentaenoic acid.

TABLE 4-5

Relationship of ex vivo prostaglandin production to tissue arachidonic acid content [1]

Prostaglandins	Correlation Coefficient (r)	Level of Significance (*P*)
PGE_2	0.71	0.0003
6-keto-PGF1α	0.73	0.0004

[1] *Ex vivo* production of prostaglandins in normal-appearing small intestine were correlated with the arachidonic acid content of normal-appearing small intestine phospholipids. The correlation coefficient (r) and significance level of the correlation were determined by correlation analysis.

TABLE 4-6

Prostaglandin formation in normal-appearing small intestine[1]

	Dietary Groups			
	OA[2] (n=5)	AA (n=5)	EPA (n=5)	AA+EPA (n=5)
Prostaglandins[2]	*ng/mg protein*			
Basal				
PGE_2	9.8±2.2[a]	12.4±2.7[a]	3.3±0.8[b]	10.2±2.0[a]
6-keto-$PGF_{1\alpha}$	14.2±0.7[a]	12.3±2.5[ab]	6.7±0.7[c]	9.9±0.8[bc]
Ex vivo production				
PGE_2	22.5±7.6[b]	53.6±7.4[a]	6.1±1.3[c]	28.0±5.0[b]
6-keto-$PGF_{1\alpha}$	44.4±11.7[b]	82.3±8.2[a]	19.4±4.0[c]	61.7±12.2[ab]

[1]Values are mean ± SEM. Transformations were performed to equalize variances prior to statistical analyses. Different superscripts within each row indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at $P < 0.05$.

[2]Abbreviations: OA: oleic acid (control), AA: arachidonic acid, EPA: eicosapentaenoic acid.

DISCUSSION

This study is the first to report that dietary EPA has anti-tumorigenic properties in *Apc*$^{Min/+}$ mice, a mouse model characterized by a germline mutation in the *Apc* tumor suppressor gene. Two other studies have also investigated the effects of dietary n-3 PUFA on tumorigenesis in mouse models with *Apc* gene defects, but with mixed results. Our results are strengthened due to the study design. Total dietary fat has a positive effect on tumor number in this mouse model (17) with potential antagonism among the n-3 and n-6 families of fatty acids (28). We controlled dietary fat composition by substituting the ethyl esters of EPA and /or AA for OA, thus any modification in tumor load would be the direct result of dietary changes of these select fatty acids. Oshima et al. (1995) observed that supplementation of DHA ethyl ester to diets of *Apc*$^{\Delta 716}$ knockout mice reduced tumor number in females, but not males. Small sample sizes could have accounted for the differences, but the type of n-3 PUFA used could have been an important factor. If EPA is the most biologically potent of the n-3 PUFA, the efficacy of DHA may depend, in part, upon the extent of its retro-conversion to EPA. However, tissue fatty acid and eicosanoid data were not presented in this study (15). In a study investigating dietary supplementation of EPA plus DHA, tumor load in *Apc*$^{Min/+}$ mice was reduced in both males and females with a more consistent effect in females (16). The major dietary difference in these two previous studies using n-3 PUFA was the inclusion of EPA in the latter.

The link between efficacy of EPA and AA metabolism is buoyed by the overwhelming evidence that NSAIDs can reduce tumor load in this animal model by 50 to 98 percent (24-27). NSAIDs are believed to be acting through the inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), the committed steps in

prostaglandin biosynthesis (25, 31, 32). Of the two identified COX isoforms, inhibition of COX-2, the inducible form, is believed to be most relevant in this model which overexpresses COX-2 in tumors (33-37). Selective inhibition of COX-2 suppresses intestinal tumor formation by 50% (35, 38). Crossing COX-2 knockout mice with *Apc* knockout mice reduces tumors by 85% (35), and dose dependent overexpression of COX-2 is associated with increased tumor number (35). COX-2 expression has also been inversely associated with apoptosis (25, 39). Dietary n-3 PUFA, like NSAIDs, are effective competitive inhibitors of COX activity, have been shown to reduce COX-2 expression (40) and increase apoptosis and differentiation in a chemically-induced colonic tumor rat model (6, 9), whereas diets high in n-6 PUFA (*viz.*, linoleic acid) increase COX-2 expression (40). AA, in particular, has been found to increase COX-2 gene expression in intestinal crypt epithelial cells (41). We have shown previously that dietary AA and EPA have an antithetic relationship on prostaglandin production *in vivo* (28), and this inverse relationship might explain the demonstrated anti-tumorigenic effect of n-3 PUFA. Consequently, in the present study, we observed characteristic reductions in prostaglandin biosynthesis following EPA supplementation and this was reversed by the addition of AA to the EPA diet. These results paralleled tumor load.

A number of studies also suggest a possible prostaglandin-independent mechanism may exist (24, 42-46). For example, Chan et al. (1998) recently reported that elevating cellular AA levels *in vitro* exerts tumor suppressor effects in HCT116 and SW480 colon cancer cell lines by enhancing ceramide-induced apoptosis, possibly explaining the anti-tumorigenicity of NSAIDs (42). While we and others have shown that dietary AA has a dramatic and potent impact on altering tissue content of AA systemically (28, 29, 47, 48), we have consistently failed to demonstrate significant reductions in

tumor number following a doubling of AA content in intestinal phospholipids of *Apc*$^{Min/+}$ mice (24). The mode of delivery of AA influences cellular distribution and utilization of this fatty acid (49, 50), therefore differing environments (i.e., *in vivo* versus *in vitro*) could explain these seemingly discordant results.

Alternatively, others have suggested an increase in lipid peroxidation may be responsible for the anti-tumorigenic effects of n-3 PUFA. It has been reported that dietary n-3 PUFA increase oxidation potential of tissues resulting in increased products of lipid peroxidation leading to tumor death (51). However, AA has a similar unsaturation index to EPA. Adding AA to the diet failed to reduce tumor load (24) and doubling the dietary unsaturation index by adding AA to the EPA diet (AA+EPA) more than doubled the tumor number (compared to the EPA group) as opposed to further reducing the tumor number as might be predicted by the above hypothesis. Therefore, although indices of lipid peroxidation were not measured, it is reasonable to conclude that lipid peroxidation may not be responsible for the reduction of intestinal tumors in the EPA-fed group.

In summary, the tightly controlled dietary design of the present study strongly reinforces previous data in other models on the anti-tumorigenicity of EPA. EPA reduced the number and size of tumors in male *Apc*$^{Min/+}$ mice and these changes were associated with reductions in tissue AA content and prostaglandin levels. AA, on the other hand, had no effect on tumor load despite increasing tissue AA content and *ex vivo* prostaglandin production. Dietary AA largely reversed changes observed with EPA alone when AA and EPA were fed concomitantly, suggesting EPA reduces tumor load, at least in part, by acting as an antagonist to AA. Although these effects appear to be correlated with prostaglandin inhibition, we cannot exclude potential involvement of prostaglandin-independent pathways.

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Part V

Selective Inhibition of Δ-6 Desaturase Impedes *Apc*-mediated Tumorigenesis

This manuscript is being prepared for publication with co-authors Michael F. McEntee, Benjamin T. Johnson, Mark G. Obukowicz, Jaime Masfarrer, Ben Zwiefel, Chun-Hung Chiu, and Jay Whelan in Cancer Letters

ABSTRACT

Arachidonic acid is an important polyunsaturated fatty acid involved in cell signaling. It is derived primarily from dietary linoleic acid, and the rate-limiting step in its biosynthesis is the initial desaturation of linoleic acid via Δ-6 desaturase. Evidence suggests that downstream metabolic products of arachidonic acid, e.g. prostaglandins, are involved in colorectal cancer, but involvement of the biosynthetic pathway of arachidonic acid has not been previously investigated. In the present study, we report on the effects of a novel selective Δ-6 desaturase inhibitor, SC-26196, on tumorigenesis in a murine models of *Apc*-mediated intestinal cancer. SC-26196 treatment resulted in 36-37% fewer tumors in two separate experiments in $Apc^{Min/+}$ mice ($P<0.05$). As expected, SC-26196 treatment also resulted in significantly higher tissue phospholipid levels of linoleic acid and lower levels of arachidonic acid. The effects on both tissue fatty acid composition and tumorigenesis were abrogated by concomitant treatment with dietary arachidonic acid, indicating that observed effects were due to interference with the biosynthetic pathway of arachidonic acid.

INTRODUCTION

Arachidonic acid (AA) is arguably the most important polyunsaturated fatty acid (PUFA) associated with membrane phospholipids. Upon stimulation, AA is released by a variety of phospholipases and is subsequently oxidized via one of a number of enzymatic pathways to form a host of bioactive lipids (e.g. eicosanoids) that mediate cell signaling and gene expression. Tissue arachidonate is derived primarily from dietary linoleic acid, the major PUFA in the U.S. diet. This conversion is regulated by Δ-6 desaturase, the first step in this metabolic pathway (1). Human Δ-6 desaturase is a 52.2 kDa membrane-

bound protein associated with the microsomal membrane fraction (1). It bears 87% homology to murine Δ-6 desaturase and it is expressed in a variety of tissues including brain, liver, lung, and heart (2). Δ-6 desaturase incorporates a double bond at the C-6 carbon of PUFA and thus, is rate limiting in the desaturation and elongation of linoleic acid to arachidonic acid (n-6 family) and α-linolenic acid to eicosapentaenoic acid (n-3 family). The importance of the Δ-6 desaturase step has yet to be investigated with regard to cancer and studies investigating this relationship are a logical extension to work already linking the arachidonic acid cascade to cancer and other chronic diseases. These previous studies have focused on the downstream products of arachidonic acid metabolism (*viz.*, inhibition of prostaglandin biosynthesis by nonsteroidal anti-inflammatory drugs)(as reviewed by Levy) (3), but none have yet directly investigated the proximal steps regulating the biosynthetic pathway of arachidonate.

We proposed, therefore, to investigate the effects of inhibiting Δ-6 desaturase on intestinal tumorigenesis and also the effects of bypassing this inhibition via the concomitant addition of dietary arachidonate (**Fig. 5-1**). SC-26196 is a highly-selective inhibitor of Δ-6 desaturase (4) and arachidonate biosynthesis (4). It was previously demonstrated in the mouse carrageenan paw edema model of inflammation that SC-26196 reduced paw edema by 50% and these effects were directly linked to its ability to inhibit arachidonic acid biosynthesis (4). Consequently, we chose to use SC-26196 in a murine model of intestinal cancer: the *Apc*$^{Min/+}$ mouse model of familial intestinal tumorigenesis. *Apc*$^{Min/+}$ mice carry a germline mutation in *Apc* and this model has been used extensively to evaluate the effects of pharmacological and nutritional intervention on intestinal tumorigenesis because of its recognized value in modeling human colorectal carcinogenesis (5). Development of colorectal cancer in humans from dysplastic crypts

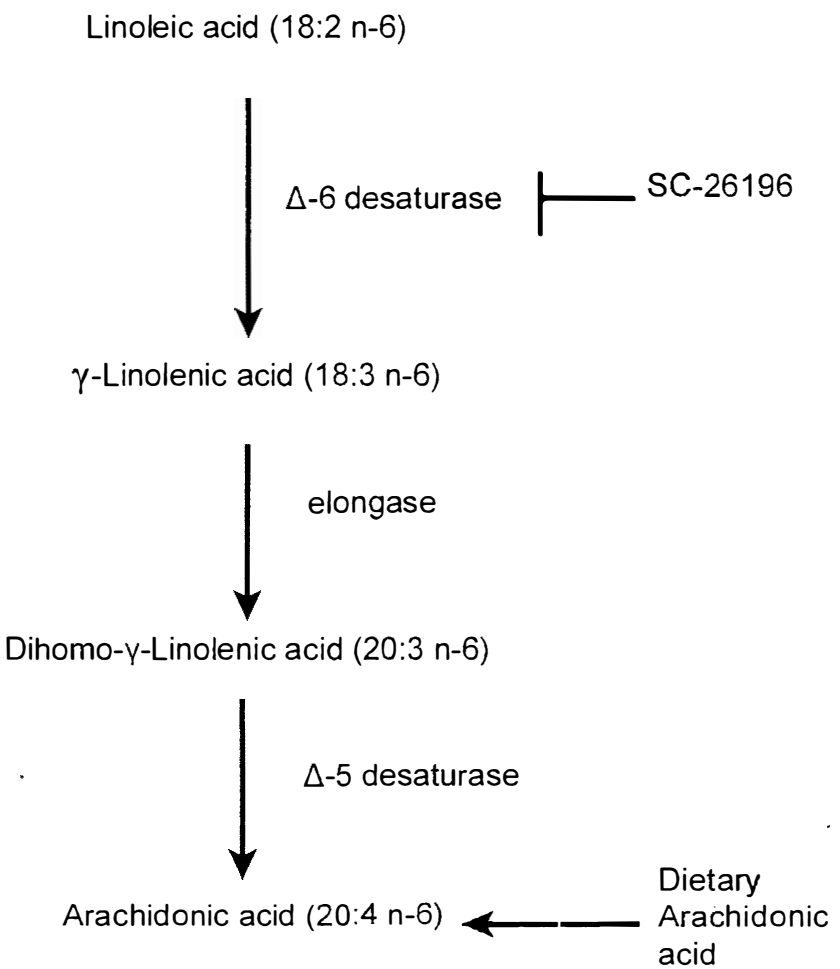

FIGURE 5-1. The selective Δ-6 desaturase inhibitor SC-26196 was utilized to investigate the effects of Δ-6 desaturase inhibition and the effects of bypassing this inhibition with the addition of dietary arachidonic acid on intestinal tumorigenesis in *Apc*$^{Min/+}$ mice.

to metastatic carcinoma involves a series of genetic mutations, the earliest often involving *APC* such that a majority of human colorectal cancers have an associated *APC* gene defect (as reviewed by Kinzler and Vogelstein) (5).

MATERIALS AND METHODS

Animals. Male C57BL/6J *Apc*$^{Min/+}$ mice (Jackson Laboratories, Bar Harbor, ME), were randomized into respective groups upon arrival. They were housed in a temperature-controlled room with a 12 h light/dark cycle and given free access to food and water. The health of the mice was checked daily. Food was withheld overnight prior to sacrifice. All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Experiment 1. Five *Apc*$^{Min/+}$ mice (~38 days of age) were treated with the selective Δ-6 desaturase inhibitor SC-26196 (100 mg/kg body weight/day) (Monsanto Co., St. Louis, MO) for 7 wk and compared to untreated control mice (n=10). The mice were fed an AIN-93G diet and SC-26196 was thoroughly mixed with the diet each week based on body weight and food intake data. All diets were divided into daily aliquots and stored under an atmosphere of nitrogen at -80°C. All mice were provided fresh food daily. Following sacrifice, intestines were rapidly removed, flushed with ice-cold saline, and dissected longitudinally. Tumor number was determined macroscopically using a dissecting microscope (X18), as previously described (6).

Experiment 2. Thirty-nine *Apc*$^{Min/+}$ mice (~38 days of age) were randomized into four dietary groups (n=9-10/group) upon arrival. Experimental groups received SC-26196 (100 mg/kg body weight/day), arachidonic acid ethyl ester (AA, 1% w/w) in place

of an equivalent amount of high-oleic acid sunflower oil (Monsanto Co., St. Louis, MO), or both SC-26196 and arachidonic acid ethyl ester (SC-26196+AA), while the control group received the base diet. All mice were fed the US17 diet (Monsanto Co., St. Louis, MO) prepared by Research Diets, Inc. (New Brunswick, NJ). The US17 diet is based on the composition of a typical Western diet and was used because it contains a high oleic acid sunflower oil "cassette" which can be eucalorically replaced by other dietary lipids (7). SC-26196 was thoroughly mixed with the diet each week based on body weight and food intake data. All diets were divided into daily aliquots and stored under an atmosphere of nitrogen at -80°C to prevent oxidation. All mice were provided fresh food daily. After treatment for 7 wk, mice were sacrificed and tumor number, size, and location were determined as in Experiment 1. Samples of normal-appearing small intestine were collected for fatty acid analysis.

Fatty acid analysis. Fatty acid methyl esters of the tissue phospholipids were prepared and analyzed as described previously (8). Briefly, tissues were homogenized in ice-cold saline and lipids were extracted with chloroform:methanol (1:2 v/v), followed by extraction (x2) with chloroform. The pooled chloroform extracts were evaporated and resuspended in a small volume of chloroform, and the phospholipids were separated by TLC on silica gel 60 HP-TLC plates (Merck, Darmstadt, Germany) with chloroform:methanol (8:1 v/v) as the solvent system. Bands corresponding to the phospholipids were scraped, dissolved in toluene, and saponified with KOH 0.5 mol/L in methanol for 8 minutes at 86°C. After acidification with HCl in methanol 0.7 mol/L, the fatty acids were extracted with hexane (x2), evaporated, and methylated with ethereal diazomethane. The fatty acid methyl esters were resuspended in hexane and analyzed using a Hewlett-Packard model 5890 series II gas chromatograph equipped with flame

ionization detector and a DB23 fused silica capillary column (0.25 mm i.d. x 30 m x 0.25 µm film; J&W Scientific, Folsom, CA). Separation was achieved by temperature programming from 160°C to 250°C at 3.5°C/minute with hydrogen as the carrier gas. The internal standard, pentadecaenoic acid (15:0) methyl ester (Avanti Polar Lipids, Alabaster, AL) was added to each sample prior to the saponification step. The fatty acid methyl esters were identified by comparison of retention times with those of known standards (Nu-Chek Prep, Elysian, MN).

Statistical analyses. Values are expressed as means ± SEM. Differences in tumor number, tumor size, and biochemical parameters were analyzed statistically by Student's t-test (one-tailed, Experiment 1) and one-way ANOVA followed by Fisher's least significant difference multiple comparison method (Experiment 2) to determine differences among groups. Tumor number data in Experiment 2 were log transformed to normalize distributions prior to statistical analysis. The Statistical Analysis System (SAS Version 6.12, SAS Institute, Inc., Cary, NC) was used to evaluate the data. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of SC-26196 treatment on tumor frequency and size in $Apc^{Min/+}$ mice. In Experiment 1 (**Fig. 5-2**) and Experiment 2 (**Table 5-1**), SC-26196 treatment resulted in 36% and 37% fewer intestinal tumors, respectively, in $Apc^{Min/+}$ mice compared to untreated controls. Dietary AA alone did not significantly alter tumor number or size compared to the control groups. However, supplementation of AA to SC-26196-treated mice normalized both tumor number and size (**Table 5-1**).

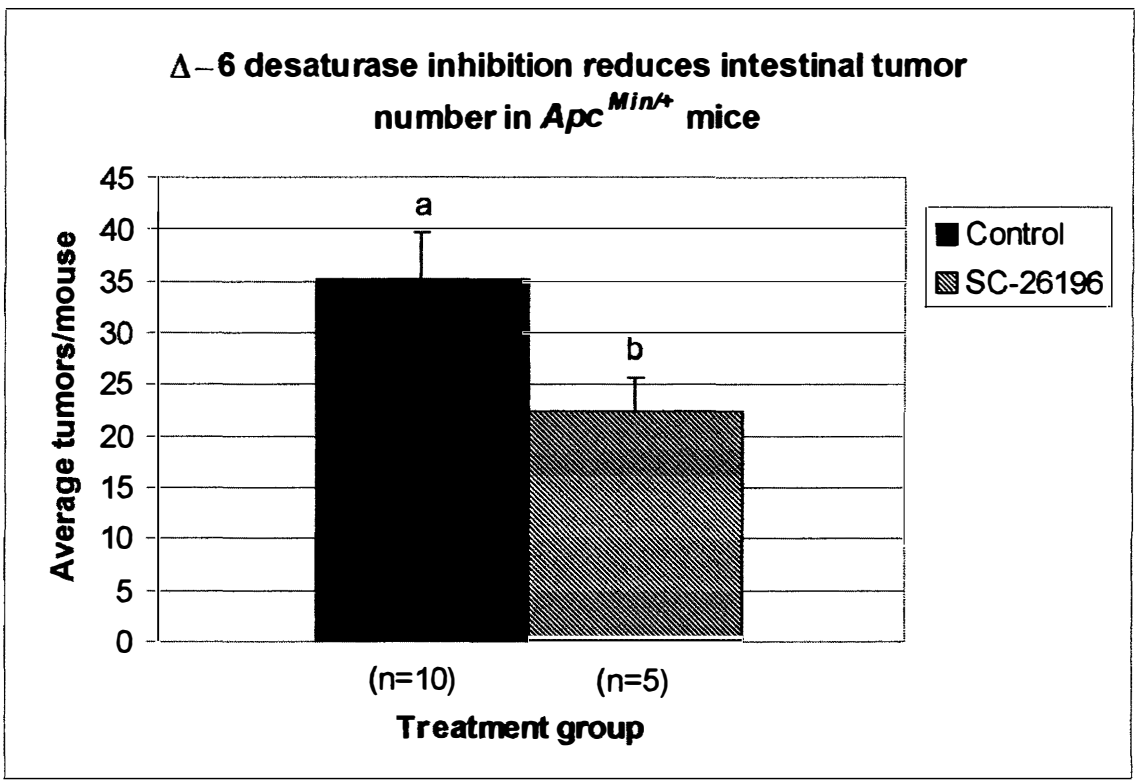

FIGURE 5-2. Differences in total tumor number between male *Apc*$^{Min/+}$ mice fed the AIN-93G control diet or the AIN-93G diet supplemented with the selective Δ-6 desaturase inhibitor SC-26196 (100 mg/kg body weight/day) for 7 wk was determined by Student's t-test (one-tailed). Values are means ± SEM. Different superscripts indicate a significant difference at $P < 0.05$.

TABLE 5-1

Number and size of intestinal tumors in $Apc^{Min/+}$ mice after dietary AA supplementation, inhibition of de novo AA biosynthesis with SC-26196, or bypassing the inhibition with SC-26196+AA[1]

	Dietary groups			
Tumor load	Control (n=10)	AA (n=10)	SC-26196 (n=10)	SC-26196+AA (n=9)
Tumors per mouse	53.5±7.1[a]	53.9±9.6[a]	33.8±5.2[b]	49.9±5.8[a]
Tumor size[2] (mm)	1.13±0.05[a]	1.12±0.04[a]	0.97±0.02[b]	1.11±0.03[a]

[1]Values are means ± SEM. Different superscripts within each row indicate a significant difference among groups by Fisher's least significant difference multiple comparison method (one-tailed) at $P < 0.05$. Tumor number data were log transformed to normalize distributions prior to statistical analysis. The control diet is Monsanto "US17" diet (Monsanto Co., St. Louis, MO, and Research Diets, Inc., New Brunswick, NJ).

[2]Average tumor size was calculated as a weighted average for each size classification and expressed as mean ± SEM.

Intestinal fatty acid composition. Substituting arachidonate ethyl ester in the diet for an equivalent amount of high oleic acid sunflower oil increased tissue phospholipid levels of arachidonate by 53% and reduced phospholipid levels of linoleate by 36% (**Table 5-2**). These changes are typical following arachidonate supplementation (6, 9, 10). Treatment with SC-26196 resulted in AA levels that were 18% lower and LA levels that were 22% higher, indicating the expected inhibition of Δ-6 desaturase. Following arachidonate supplementation to SC-26196 treated mice (SC-26196+AA), phospholipid arachidonate levels were 97% higher and linoleate levels were 46% lower as compared to the SC-26196-treated group, indicating the addition of arachidonate to the diet bypassed SC-26196-mediated inhibition of Δ-6 desaturase.

DISCUSSION

SC-26196 has been shown to be an effective selective inhibitor of Δ-6 desaturase both *in vitro* and *in vivo* (4). It is >1,000 times more selective for Δ-6 desaturase compared to either Δ-5 desaturase or Δ-9 desaturase with an IC_{50} of 0.2 μM. SC-26196-treated mice are unable to detectably convert ^{14}C-LA to fatty acids downstream of Δ-6 desaturase including γ-linolenic acid (18:3 n-6), dihomo-γ-linolenic acid (DGLA, 20:3 n-6) and AA, whereas desaturation of ^{14}C-DGLA to AA is not inhibited (4). Similarly, the fatty acid composition of phospholipids in *Apc*$^{Min/+}$ mice (present study) also suggests that SC-26196 is inhibiting Δ-6 desaturase with significant reductions in AA content and increases in LA. This inhibition is circumvented with the addition of dietary arachidonate (**Fig. 5-1**).

We report for the first time that selective inhibition of Δ-6 desaturase inhibits intestinal tumorigenesis *in vivo*. These data suggest that inhibition of arachidonate

TABLE 5-2

Fatty acid composition of mouse small intestinal phospholipids after dietary AA supplementation, inhibition of de novo AA biosynthesis with SC-26196, or bypassing the inhibition with SC-26196+AA

	Dietary groups			
	Control (n=10)	AA (n=10)	SC26196 (n=10)	SC26196+AA (n=9)
Fatty acid	*mol% of total fatty acids*			
18:2 (n-6)	21.48±0.37[b]	13.68±0.44[c]	26.29±0.56[a]	14.16±0.27[c]
20:4 (n-6)	18.83±0.26[c]	28.89±0.79[b]	15.44±0.40[d]	30.40±0.36[a]

[1]Values are means ± SEM. Different superscripts within each row indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at $P < 0.05$. The control diet is Monsanto "US17" diet (Monsanto Co., St. Louis, MO, and Research Diets, Inc., New Brunswick, NJ).

biosynthesis attenuates intestinal tumorigenesis and further support the concept that arachidonic acid is important in maintaining tumor integrity. Regulating availability of arachidonate appears to be critical for downstream signaling events that regulate tumorigenesis *in vivo*. For example, crossing $cPLA_2$ knockout mice with $Apc^{Min/+}$ mice suppresses intestinal polyp development by 83% (11). $cPLA_2$ is the rate-limiting step in the release of arachidonate from membrane phospholipids and in prostaglandin biosynthesis, and NSAIDs have been proposed to be anti-tumorigenic, in part, by limiting the supply of arachidonate via down-regulation of $cPLA_2$ (12). Interestingly, enriching tissues with arachidonate did not augment tumor load, thus reproducing our previously published results (6, 10, 13)

While the lower tumor number in the SC-26196-treated mice suggests Δ-6 desaturase involvement, this interconnection is strengthened by the fact that concomitant arachidonate supplementation abrogates the anti-tumorigenic efficacy of SC-26196. Using a similar dietary design, we previously showed that dietary arachidonate also reverses the anti-tumorigenic efficacy of dietary n-3 PUFA in $Apc^{Min/+}$ mice (10). N-3 PUFA, like SC-26196, are inhibitors of Δ-6 desaturase (14) and, furthermore, they compete with arachidonic acid for incorporation into membrane phospholipids (6, 9, 10). Treating $Apc^{Min/+}$ mice with diets rich in n-3 PUFA reduces arachidonate content in intestinal phospholipids and also reduces tumor number by ~50%. Similar to findings of the present study, these effects are reversed by concomitant supplementation with dietary arachidonic acid (10). This suggests that n-3 PUFA act via antagonism of arachidonic acid metabolism, perhaps in part, via a mechanism similar to that of SC-26196. Additionally, another experiment with a parallel dietary design demonstrated that SC-26196 reduced paw edema in a carrageenan paw edema model of inflammation, and

these effects were likewise reversed by dietary arachidonate (4). While the consistency of these results is compelling, we cannot exclude the possibility that SC-26196 is acting by a mechanism independent of Δ-6 desaturase inhibition. However, this seems unlikely given the fact that dietary archidonate appears to negate the anti-tumorigenic and anti-inflammatory effects of SC-26196.

These results do not attempt to explain the downstream signaling whereby SC-26196 attenuates tumorigenesis, but only reveal the fact that its effects are mediated through inhibition of arachidonic acid biosynthesis. One possibility is the attenuation of prostaglandin biosynthesis due to decreased substrate availability. Multiple studies using mouse models with *Apc* defects have clearly established the involvement of cyclooxygenase and prostaglandins in intestinal tumorigenesis (15-19).

In summary, direct inhibition of Δ-6 desaturase with the selective inhibitor SC-26196 attenuates tumorigenesis in $Apc^{Min/+}$ mice. The mechanism of action most likely involves attenuation of arachidonate biosynthesis, as the anti-tumorigenic effect of SC-26196 was nullified by bypassing Δ-6 desaturase inhibition with concomitant dietary arachidonate supplementation.

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PART VI

Prostaglandin E_2 Protects Intestinal Tumors from Nonsteroidal Anti-inflammatory Drug-induced Regression in *Apc*$^{Min/+}$ Mice

This manuscript has been prepared for publication with co-authors Michael F. McEntee, Brian Jull, and Jay Whelan in Carcinogenesis

ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are anti-tumorigenic in humans as well as animal models of intestinal tumorigenesis, such as the *Apc*$^{Min/+}$ mouse. NSAIDs inhibit cyclooxygenase (COX) isozymes, which are responsible for the committed step in prostaglandin (PG) biosynthesis, and this has been considered the primary mechanism by which NSAIDs exert their anti-tumorigenic effects. However, mounting evidence suggests the existence of COX-independent mechanisms. In the present study, we attempted to clarify this issue by treating *Apc*$^{Min/+}$ mice with NSAIDs (piroxicam and sulindac, 0.5 and 0.6 mg/mouse/day respectively) for 6 days and concomitantly bypassing COX inhibition by treatment with the PGE receptor agonists (EP*R*-A) 16,16-dimethyl-PGE_2 and 17-phenyl-trinor-PGE_2 (10 µg each, three times daily) administered via gavage and/or i.p. routes. Treatment with either piroxicam or sulindac resulted in 95% and 52% fewer tumors, respectively, and a higher ratio of apoptosis:mitosis in tumors from sulindac-treated mice as compared to controls, and these effects were attenuated by concomitant EP*R*-A treatment, suggesting PGE_2 involvement in maintenance of tumor integrity. Immunologic sequestration of PGE_2 with an anti-PGE_2 monoclonal antibody likewise resulted in 33% fewer tumors in *Apc*$^{Min/+}$ mice relative to untreated controls, further substantiating a role for PGE_2. Overall, our results suggest that NSAIDs exert their anti-tumorigenic effects, in part, via interference with PGE_2 biosynthesis and subsequent downstream signaling.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer deaths in the United States with 56,785 deaths reported in 1998 (1). Over the last several years, the *Apc*$^{Min/+}$

mouse has been extensively used to evaluate the effects of pharmacological and nutritional intervention on intestinal tumorigenesis because of its recognized value in modeling human colorectal carcinogenesis (2). Development of colorectal cancer in humans from dysplastic crypts to metastatic carcinoma involves a series of genetic mutations, the earliest often involving *APC* (3). Individuals with familial adenomatous polyposis (FAP), like $Apc^{Min/+}$ mice, possess a germline mutation in *APC* and mutational damage or loss of the wild type allele initiates intestinal tumor formation (4). Although FAP accounts for <1% of all human colorectal cancer cases, somatic mutations resulting in loss of full length *APC* protein also occur early in spontaneous forms of the disease (5, 6) indicating an *APC* defect is associated with a majority of human colorectal cancers (5, 7, 8).

Multiple studies using the $Apc^{Min/+}$ mouse model have clearly established the anti-tumorigenic efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs), inhibitors of COX-1 and COX-2, isozymes responsible for the committed step in prostaglandin biosynthesis. The COX-inhibitory effects of NSAIDs have been considered key to their anti-tumorigenic efficacy and this hypothesis is supported by several lines of evidence. COX-2, the inducible isoform, is overexpressed in intestinal tumor tissue but not normal intestinal tissue of both humans and $Apc^{Min/+}$ mice (9-11). Corresponding to the overexpression of COX-2, PGE_2, the COX product of arachidonic acid (20:4 n-6) metabolism, is elevated in human colonic tumors (12, 13) and in intestinal tumors from $Apc^{Min/+}$ mice compared to normal intestinal tissue (14). Furthermore, inhibition of both COX-1 and COX-2 by n-3 polyunsaturated fatty acids and non-selective inhibitors, including the NSAIDs sulindac, indomethacin, piroxicam, and aspirin, reduces tumor number in $Apc^{Min/+}$ mice by 44-96% (14-21) and selective inhibition of COX-2 reduces

tumor number by 52-71%(22, 23). Likewise, crossing COX-2 knockout mice with *Apc*$^{\Delta 716}$ mice or *Apc*$^{Min/+}$ mice reduced tumors by ~85% (24, 25) and crossing *Apc*$^{Min/+}$ mice with COX-1 knockouts similarly reduced tumor multiplicity by 77% (24).

Despite these supportive data, mounting evidence suggests that NSAIDs may also work via COX-independent mechanisms. For example, *S*-flurbiprofen, a nonselective COX inhibitor, and its inactive enantiomer (*R*-flurbiprofen) were equally effective in reducing tumor number in *Apc*$^{Min/+}$ mice (26). Additionally, NSAIDs have been shown to modulate cell proliferation and cell death in colon cancer cells lacking COX, suggesting that not all NSAID effects are based on COX inhibition (27-29). Multiple COX-independent mechanisms have been investigated to date including those involving 15-lipoxygenase-1 (30), ceramide (31, 32), p21 (33), β-catenin (34)(28, 35), peroxisome proliferator-activated receptors (PPARs) (36), IκB kinase (37), and cGMP phosphodiesterase (38). While many of these alternative mechanisms may involve signaling pathways related to COX inhibition and prostaglandin biosynthesis, these links have yet to be definitively established. Thus, in order to more clearly establish the role of NSAIDs and prostaglandins in the maintenance of tumor integrity, we endeavored to attenuate NSAID-induced regression of intestinal tumors through a series of "add-back" experiments involving PGE receptor agonists (EP*R*-A) and by systemically sequestering PGE_2 using an anti-PGE_2 antibody. To do this, we capitalized on previous results demonstrating that NSAIDs (i.e. piroxicam and sulindac) could eliminate up to 95% of preexisting tumors within 6 days (14, 21) and that i.p. administration of the EP*R*-A 16,16-dimethyl PGE_2 had direct effects on the stem cell population in small intestinal crypts of mice (39). The results of these *in vivo* experiments will demonstrate that PGE_2

is important in maintaining tumor integrity and that PGE_2 may also be involved in tumor regression.

MATERIALS AND METHODS

Animals. Male C57BL/6J *Apc*$^{Min/+}$ mice (Jackson Laboratories, Bar Harbor, ME), were obtained at 38-45 days of age. They were housed in a temperature-controlled room with 14 h periods of light and 10 h periods of darkness and given free access to food and water. The health of the animals was checked daily. Food was withheld overnight prior to sacrifice. All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Diets. Diets for Experiments 1-3 were composed of purified AIN-93G powder diet (Dyets, Inc., Bethlehem, PA). Experimental diets containing NSAIDs were prepared daily by thoroughly mixing piroxicam (Sigma, St. Louis, MO) or sulindac (Sigma, St. Louis, MO) with the control diet. Diets were stored at -20°C and all mice were provided fresh food daily. Food consumption was monitored daily and body weights were recorded weekly.

Experimental design.

Experiment 1: Mice (n=23) were maintained on the AIN-93G diet until 78-79 days of age at which time they were randomly assigned to one of four groups (control, EP*R*-A, piroxicam, or piroxicam + EP*R*-A). Groups receiving piroxicam (0.5 mg/mouse/day) were pair-fed to ensure equivalent dosing. The EP*R*-A 16,16-dimethyl-PGE_2 and 17-phenyl-trinor-PGE_2 (Cayman Chemical, Ann Arbor, MI), 10 µg each in sterile PBS, or vehicle were administered in two daily i.p. injections (0800, 1600) and once daily via

gavage feeding (1200) to maximize exposure to the gastrointestinal tract over a six-day period. They were sacrificed at 85-86 days of age by cervical dislocation and tumor number, size, and location were determined as previously described (14).

Experiment 2: Mice (n=30) were maintained on the AIN-93G diet until 80-81 days of age at which time they were randomly assigned to one of four groups (control, EP*R*-A, sulindac, or sulindac + EP*R*-A). Groups receiving sulindac (0.6 mg/mouse/day) were pair-fed to ensure equivalent dosing. The EP*R*-A 16,16-dimethyl-PGE_2 and 17-phenyl-trinor-PGE_2 (Cayman Chemical, Ann Arbor, MI), 10 µg each in sterile PBS, or vehicle were administered every 8 h via i.p. injection over a six-day period. Mice were sacrificed at 86-87 days of age and treated as in Experiment 1.

Experiment 3: Mice (n=17) were maintained on the AIN93G diet until 82 days of age at which time they were randomly assigned to one of two groups (control or PGE_2 antibody). Control mice received the MOPC21 mouse IgG1 purified immunoglobulin (Sigma, St. Louis, MO) (280 µg in 280 µl) that was filter-sterilized (0.45 µm filter) and administered daily via i.p. injection on days 82-85. The anti-PGE_2 monoclonal antibody, 2B5 (Monsanto Co., St. Louis, MO) (40) (283 µg/day in 250 µl sterile PBS), was administered daily via i.p. injection on days 82-85. All mice were sacrificed on day 87 and treated as in Experiments 1 and 2.

Measurement of apoptosis and mitosis. Tumors were immediately placed in 10% neutral buffered formalin. After 8-10 hours of fixation tissues were routinely processed into paraffin and 4 µm hematoxylin and eosin (H&E) stained sections prepared for histologic examination. Neoplastic epithelial cells undergoing apoptosis or mitosis were identified under 400X magnification in H&E stained sections of small intestinal tumors according to well-characterized morphologic criteria and without prior

knowledge of the study group. The number of apoptotic or mitotic events were simultaneously enumerated per 1000 cells for each tumor and recorded as:

[number of apoptotic or mitotic cells ÷ total number of cells counted] x 1000.

Statistical analyses. Values are expressed as means ± SEM. With the exception of tumor number in Experiment 1, data for Experiments 1 and 2 (differences in tumor number, tumor size, and mitotic index) were analyzed statistically by one-way ANOVA followed by Fisher's least significant difference multiple comparison method to determine differences among groups. Mitotic index data were transformed (log[Y+1]) to normalize sample distributions prior to analysis. Tumor number in Experiment 1, apoptotic index, and apoptosis:mitosis ratio were analyzed by the Kruskal-Wallis (rank sums) test with post hoc Bonferroni adjustment to control the experimentwise error rate. Student's t-test was used to analyze data in Experiment 3. The Statistical Analysis System (SAS Version 6.12, SAS Institute, Inc., Cary, NC) was used to evaluate the data. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of piroxicam, sulindac, EPR-A, and PGE_2 antibody treatment on tumor burden. Mice treated with piroxicam and sulindac had 95% and 52% fewer intestinal tumors, respectively, as compared to control mice and this effect was partially attenuated by concomitant EP*R*-A treatment ($P < 0.05$) (**Tables 6-1** and **6-2**). Antagonism of PGE_2 with 2B5 anti-PGE_2 antibody treatment also resulted in 33% fewer tumors compared to controls ($P < 0.05$) (**Table 6-3**). Small intestinal erosions and/or ulcers were commonly identified with a dissecting microscope in the mucosa of piroxicam- and, to a lesser extent, sulindac-treated mice. In contrast, there was no

TABLE 6-1

Intestinal tumor load in $Apc^{Min/+}$ mice treated with and without piroxicam +/-EPR-A

	Treatment group			
	Control (n=6)	EP*R*-A (n=5)	Piroxicam (n=5)	Piroxicam+EP*R*-A (n=6)
Tumor load[1]				
Tumors/mouse	48.8±6.2[a]	30.0±6.5[a]	2.4±0.8[b]	19.3±4.8[a]
Tumor size[2] (mm)	1.11±0.06[a]	0.99±0.04[ab]	1.03±0.12[ab]	0.88±0.05[b]

[1] Values are means ± SEM.

[2] Average size (mm) was calculated as a weighted average and expressed as mean±SEM.

[a,b] Different superscripts within each row indicate a significant difference among groups by ANOVA followed by Fisher's least significant difference multiple comparison method (tumor size) or Kruskal-Wallis with Bonferroni adjustment (tumors/mouse) at $P < 0.05$.

TABLE 6-2

Intestinal tumor load in $Apc^{Min/+}$ mice treated with and without sulindac +/- EPR-A

	Treatment group			
	Control (n=7)	EP*R*-A (n=7)	Sulindac (n=8)	Sulindac+EP*R*-A (n=8)
Tumor Load[1]				
Tumors/mouse	46.0±6.7[a]	38.0±2.6[ab]	22.1±5.8[b]	38.4±6.2[a]
Tumor size[2] (mm)	1.30±0.05[a]	1.07±0.06[b]	0.97±0.04[b]	1.10±0.04[b]

[1] Values are means ± SEM.

[2] Average size (mm) was calculated as a weighted average and expressed as mean±SEM.

[a,b] Different superscripts within each row indicate a significant difference among groups by ANOVA followed by Fisher's least significant difference multiple comparison method at $P < 0.05$. Tumors/mouse in mice receiving sulindac is significantly different from sulindac+EP*R*-A at $P=0.046$. The level of significance for tumors/mouse in mice receiving sulindac vs. those receiving EP*R*-A is $P=0.059$.

TABLE 6-3

Intestinal tumor load in $Apc^{Min/+}$ mice treated with the anti-PGE_2 mAb 2B5 compared to MOPC-21 control antibody

	Treatment group[1]	
	MOPC-21 (n=9)	2B5 (n=8)
Tumor load[1]		
Tumors/mouse	58.6±6.0[a]	39.0±6.3[b]
Tumor size[2] (mm)	1.14±0.04[a]	1.17±0.04[a]

[1] Values are means ± SEM.

[2]Average size (mm) was calculated as a weighted average and expressed as mean±SEM.

[a,b] Different superscripts within each row indicate a significant difference among groups by Student's t-test at $P < 0.05$.

evidence of such damage in the other treatment groups, particularly those treated with a NSAID plus EP*R*-A. Select small intestinal tumors in the sulindac and piroxicam treatment groups contained histologic evidence of regression, as previously described (14).

Comparison of apoptosis and mitosis in tumors. In comparison to controls, the mean apoptotic index was significantly higher in tumors from sulindac-treated mice, but the addition of EP*R*-A (sulindac + EP*R*-A) significantly attenuated sulindac's ability to induce apoptosis (**Fig. 6-1A**). The EP*R*-A alone had no impact on the apoptotic index. In contrast, the mitotic index was significantly lower in tumors from sulindac-treated mice as compared to controls and EP*R*-A treatment (sulindac+EP*R*-A) attenuated this response (**Fig. 6-1B**). Overall, tumors from the control group had an apoptosis:mitosis ratio of 0.56 (a ratio of less than one is consistent with positive tumor growth), and this ratio increased to 2.52 with sulindac treatment (**Fig. 6-1C**). The addition of EP*R*-A (sulindac+EP*R*-A) attenuated sulindac's impact on this ratio (to 1.13).

DISCUSSION

Many NSAIDs are clearly anti-tumorigenic in the *Apc*$^{Min/+}$ mouse model. Although these anti-tumorigenic effects have largely been ascribed to inhibition of prostaglandin biosynthesis, recent evidence suggests multiple mechanisms may be involved. In order to more clearly establish the role of NSAIDs and prostaglandins (particularly PGE_2) in maintaining tumor integrity, we circumvented NSAID-induced COX inhibition with concomitant EP*R*-A administration in *Apc*$^{Min/+}$ mice. In the first experiment, mice treated simultaneously with EP*R*-A and piroxicam had an 8-fold greater tumor number than those treated with piroxicam alone, indicating that PGE_2 is important in maintaining

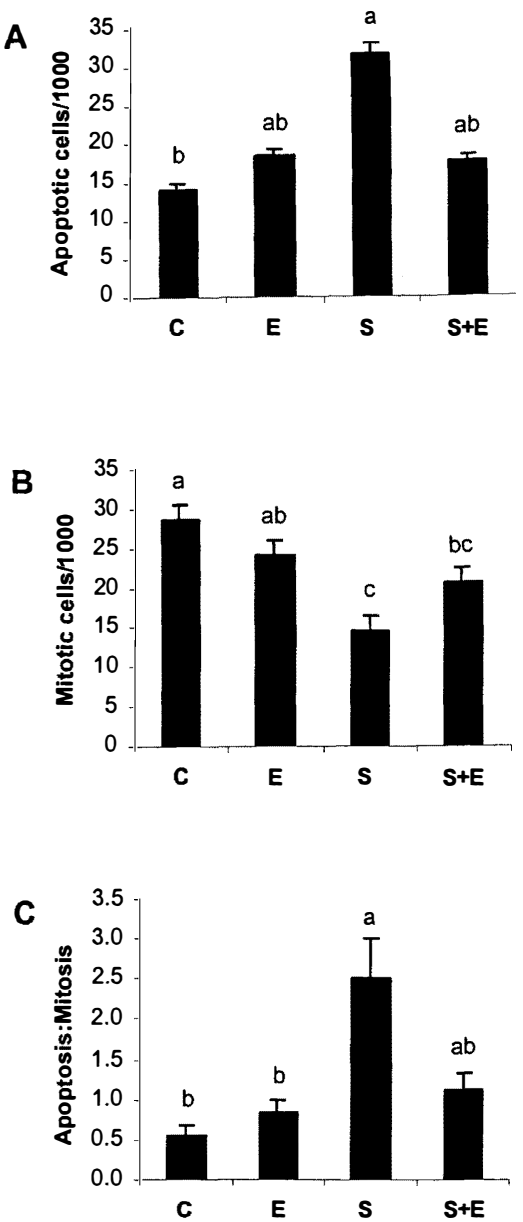

FIGURE 6-1. Effect of sulindac (S), EP*R*-A (E), and sulindac+EP*R*-A (S+E) compared to control (C) on (**A**) apoptosis, (**B**) mitosis, and (**C**) apoptosis:mitosis in tumors of $Apc^{Min/+}$ mice as determined histologically following hematoxylin and eosin staining. Different superscripts connote differences among groups at $P<0.05$.

intestinal tumor integrity and that its reduction accounts, at least in part, for the anti-tumorigenic effects of NSAIDs. Treatment with a dose of sulindac designed to yield incomplete tumor regression verified results observed with piroxicam and also allowed for subsequent tumor tissue analysis. Histologic evaluation of tumors from sulindac-treated mice indicated that PGE_2 modulates apoptosis and proliferation of neoplastic cells in intestinal tumors *in vivo* (**Fig. 6-1**). Whether this is a direct effect on the epithelium, disruption of paracrine signaling, or secondary to stromal changes remains to be determined. However, given that COX-2 is not expressed by epithelial cells, any direct effect would have to be independent of COX-2.

In a follow-up experiment, we confirmed the importance of PGE_2 in tumorigenesis by administering an antibody (2B5) that neutralizes PGE_2 *in vivo* (40). If NSAIDs induce tumor regression by reducing PGE_2 formation, then immunologic sequestration of PGE_2 should have a similar effect. Accordingly, administration of 2B5 to $Apc^{Min/+}$ mice with preexisting tumors resulted in significantly fewer tumors relative to controls following four days of treatment. Similarly, Stolina et al. observed attenuated tumor growth in mice bearing Lewis lung carcinoma xenografts following treatment with 2B5 along with a concomitant decrease in tissue PGE_2 levels (41). We failed to see differences in PGE_2 in our tissue samples (data not shown), most likely due to the length of time between administration of the final dose of 2B5 and time of sacrifice (48 h). These experiments suggest that PGE_2 mediates intestinal tumorigenesis and may be required for the maintenance of tumor integrity.

PGE_2 evokes its cellular responses via one or more of the four EP receptors (EP1-EP4) and the EP*R*-A used in this study were ligands for all receptor subtypes. Watanabe has shown that selective antagonism of EP1 results in 44% fewer tumors in

Apc$^{Min/+}$ mice (42), suggesting EP1 may be an important receptor in mediating the effects of PGE_2 in intestinal tumorigenesis. Involvement of the other EP receptors is unclear, but it seems less likely that EP3 is involved. Although EP3 is strongly expressed in myenteric ganglia and weakly expressed in longitudinal smooth muscle throughout the murine intestinal tract (43), development of aberrant crypt foci was not different in C57BL/6J EP3-/- mice compared to controls following azoxymethane treatment, whereas targeted mutations of EP1 attenuated development of azoxymethane-induced lesions in EP1-/- mice (42). EP2 is not known to be expressed in murine small intestine and therefore also seems unlikely to be a factor (43). However, EP4 and EP1 are expressed in the intestines of mice, with EP4 being highly expressed in epithelial cells (43)and thus are potential candidate receptors for the effects observed with the EP*R*-A. We used an admixture of two stable analogues of PGE_2 capable of mimicking the actions of endogenous PGE_2. Because of the putative involvement of EP1, we used the EP1/EP3 receptor agonist 17-phenyl-trinor-PGE_2. We also used 16,16-dimethyl-PGE_2 (EP2/EP3/EP4 agonist) (44) because i.p. administration has previously been shown to partially reverse indomethacin-induced reduction in survival of small intestinal crypt stem cells, the primary site for intestinal tumorigenesis in this model (39). There have been no previous reports on the effects of 17-phenyl-trinor-PGE_2 administered either i.p. or orally.

An unexpected enigma in our data is the apparent anti-tumorigenic effect of EP*R*-A (control vs. EP*R*-A: pooled data, P=0.06) that seems antithetical to its pro-tumorigenic effects when co-administered with either piroxicam or sulindac. It is possible that activation of the various EP receptors may differentially modulate tumorigenesis such that some receptors promote tumor growth while others promote tumor regression. For

example, Lehnert et al. reported that 16,16-dimethyl-PGE_2 significantly attenuated carcinogen-induced small intestinal tumors in rats (45). Moreover, it was recently reported that 16,16-dimethyl-PGE_2 reduced tumor number in $Apc^{Min/+}$ mice by 20-50% following i.p. administration three times per week for twelve weeks (46). This is in agreement with research linking increases in cAMP levels and protein kinase A activity to alterations in proliferation and differentiation in several cancer cell lines including an anti-proliferative effect on some colon cancer cell lines, both *in vitro* and *in vivo* (47, 48). Additionally, activation of EP2, EP3, or EP4 receptors has been associated with cAMP-mediated growth inhibition of B lymphocytes, NIH-3T3 cells, and mesangial cells *in vitro* whereas 17-phenyl-trinor-PGE_2 or other EP1 agonists stimulated proliferation (49-51). Perhaps stimulation of the EP1 receptor helps to maintain tumor integrity while activation of one or more of the other EP receptors, i.e. EP4, is involved in the attenuation of tumor number in this model. Therefore, our EP*R*-A mixture containing both an EP1/EP3 agonist (17-phenyl-trinor-PGE_2) and an EP2-4 agonist (16,16-dimethyl-PGE_2) may account for our seemingly contradictory results.

In summary, inhibition of PGE_2 biosynthesis accounts, at least in part, for the anti-tumorigenicity of NSAIDs. Furthermore, we show that NSAID treatment results in a higher apoptosis:mitosis ratio *in vivo* and this effect is mediated, in part, by inhibition of PGE_2 biosynthesis. Because the EP*R*-A mixture used for these studies contained ligands for all four EP receptors, we are unable to definitively ascribe the results to any one receptor or combination of receptors. However, previous research on receptor expression patterns and roles of select subtypes along with the data presented in this paper suggest that EP1 may be primarily responsible for the proliferative effects of PGE_2 in intestinal tumorigenesis. Further investigation will be required to conclusively

determine which EP receptor subtype(s) are responsible for the observed effects and whether these might prove to be dichotomous.

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PART VII

Summary and Conclusions

The research I have presented demonstrates that certain dietary fatty acids are able to reduce intestinal tumorigenesis in the *Apc*$^{Min/+}$ mouse, a unique model bearing many similarities to human colorectal cancer, and these effects are due, in part, to interference with arachidonic acid and/or its metabolism. While dietary cLA, GLA, and ALA have no effect on tumorigenesis in this model, SDA and EPA result in ~50% fewer tumors, suggesting that bypassing the rate-limiting Δ-6 desaturase step is critical for anti-tumorigenic efficacy. The efficacy of SDA may, in fact, be due to its rapid conversion to EPA, and the apparent intermediate efficacy of DHA may likewise be due to its partial retroconversion to EPA. These experiments show that the anti-tumorigenic effect of EPA is due to interference with arachidonic acid or its metabolism, as EPA supplementation reduces arachidonic acid content of intestinal phospholipids, PGE_2 levels, and tumor number, and concomitant supplementation with dietary arachidonic acid abrogates these effects. A role for arachidonic acid was confirmed by experiments showing that inhibition of *de novo* arachidonic acid biosynthesis with a selective Δ-6 desaturase inhibitor significantly reduces tumorigenesis and this effect is reversed by bypassing the inhibition with concomitant dietary arachidonic acid supplementation. Finally, my data show that PGE_2 is a mediator of intestinal tumorigenesis in this model and this may explain, in part, the anti-tumorigenic efficacy of NSAIDs as well as n-3 PUFA.

These findings have important implications for the prevention of colorectal cancer in humans. n-6 PUFA are the predominant PUFA in the Western diet totaling ~15 g/day, and intake of n-3 PUFA that bypass the Δ-6 desaturase step (primarily EPA and DHA) is only ~200 mg/day. My data suggest that increasing dietary intake of these n-3 PUFA relative to intakes of n-6 PUFA, in particular arachidonic acid, may be

important in minimizing risk of colorectal cancer. These recommendations translate into consuming more fish and shellfish, which are rich in EPA and DHA, and consuming less meats including beef and pork, which are relatively low in n-3 PUFA. In conclusion, minimizing tissue arachidonic acid levels and prostaglandin formation by elevating dietary intake of n-3 PUFA, particularly SDA or EPA, or by use of chemotherapeutic agents such as NSAIDs, is a promising means of minimizing colorectal cancer risk.

VITA

Melissa Hansen Petrik was born in Milwaukee, Wisconsin, on April 26, 1967. She graduated from Faribault Senior High School in Faribault, Minnesota, in June 1985, after which she entered South Dakota State University in Brookings, South Dakota. She graduated from SDSU in May 1990 with a Bachelor of Science in dietetics and minors in journalism and chemistry. She entered the combined dietetic internship and master's degree program at Case Western Reserve University and University Hospitals of Cleveland in Cleveland, Ohio, in August 1990 and graduated from CWRU with a Master of Science degree in nutrition in January 1992. She became a registered dietitian in 1992 and worked as a clinical dietitian and nutrition support dietitian at New Hanover Regional Medical Center in Wilmington, North Carolina, until August 1996. At that time, she returned to her studies, now at the University of Tennessee in Knoxville, Tennessee, to pursue her doctorate in nutrition. Following completion of this degree in May 2001, Melissa will stay on in an academic position in the Department of Nutrition at the University of Tennessee.