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I am submitting herewith a dissertation written by Chun-Hung Chiu entitled "The effect of modifying eicosanoid biosynthesis on tumor load in the *Min*/+ mouse." I have examined the final 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.

Dr. Jay Whelan, Major Professor

We have read this dissertation
and recommend its acceptance:

Dr. James Bailey

Dr. Roger Carroll

Dr. Naima Moustaid Moussa

Dr. Michael Zemel

Accepted for the council:

Associate Vice Chancellor and
Dean of The Graduate School

# The Effect of Modifying Eicosanoid Biosynthesis on Intestinal Tumor Load in the *Min*/+ Mouse

A Dissertation
Presented for the
Doctor of Philosophy
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Chun-Hung Chiu
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DEDICATION

This dissertation is dedicated to my parents, Mr. Chung-Chi Chiu and Mrs. Chun-Wei Shih Chiu, for their love, support and understanding, and for always telling me that I could accomplish anything I set my mind to.

I love you both.

謹將這份論文獻給我的父母 —

邱清吉先生和邱施純娓女士

感謝他們無盡的慈愛，支持與瞭解，並且總是勉勵我

「世上無難事，只怕有心人」。

爸媽，我愛您們

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## ABSTRACT

Several lines of evidence strongly link prostaglandins (PGs) and leukotrienes (LTs) to cancers of the intestine. Epidemiological studies showed aspirin and aspirin-like compounds reduced the relative risk of intestinal cancer in humans by 40-50%. Nonsteroidal anti-inflammatory drugs (NSAIDs) can cause regression of intestinal tumors in humans and laboratory animals as well. The precise mechanism of antitumor effect of these NSAIDs is uncertain, but it has been presumed the effect is due to their ability to inhibit prostaglandin biosynthesis. This dissertation is designed to investigate the effect of modifying eicosanoid biosynthesis by NSAIDs and dietary manipulation on tumor load in a mouse model (*Min*/+) containing a germline defect of the *APC* gene. *Min*/+ mice were treated with sulindac (320 ppm), aspirin (400 ppm) and indomethacin (9 ppm) to inhibit and dietary arachidonic acid (1.5 wt %) to augment eicosanoid biosynthesis. Sulindac treatment reduced tumor number by > 90%, with the recurrence of tumor load following removal of treatment. In mice with established tumors, sulindac reduced tumor number by 28% and 75% after 2 and 4 days treatment, respectively. Surprisingly, sulindac treatment did not alter the levels of eicosanoids. Both indomethacin and aspirin are potent inhibitors of prostaglandins; however, only indomethacin significantly reduced the tumor number. Elevating prostaglandin $E_2$ by 44% following dietary arachidonic acid supplementation had no effect on tumor load. In summary, the data indicate a discordance between the impact of different NSAIDs on eicosanoid biosynthesis and their antitumor activity.

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# ABBREVIATIONS

| | |
|---|---|
| Arachidonic acid | AA |
| Linoleic acid | LA |
| Eicosapentaenoic acid | EPA |
| Docosahexaenoic acid | DHA |
| Polyunsaturated fatty acid | PUFA |
| Nonsteroidal anti-inflammatory drugs | NSAIDs |
| Prostaglandin H synthase | PGHS |
| Cyclooxygenase | COX |
| Thromboxane $A_2$ | $TXA_2$ |
| Prostaglandin $E_2$ | $PGE_2$ |
| Prostacyclin $I_2$ | $PGI_2$ |
| Leukotriene $B_4$ | $LTB_4$ |
| Phospholipid | PL |
| Phospholipase $A_2$ | $PLA_2$ |
| Familial adenomatous polyposis | FAP |
| Multiple intestinal neoplasia | Min |
| Adenomatous polyposis coli | APC |
| Ethylnitrosouria | ENU |
| Hereditary nonpolyposis colorectal cancer | HNPCC |
| Glycogen synthase kinase 3β | GSK 3β |
| Modifier of Min1 | Mom1 |

# PART I

# INTRODUCTION

Cancer is the second leading cause of death in the United States. Colorectal cancer represents the second leading cause of death from all cancers and accounts for a third of the estimated new cancer cases (1). The formation of eicosanoids (prostaglandins and leukotrienes) derived from arachidonic acid (AA, 20:4n-6) have been strongly linked to cancer of the intestines. It is hypothesized that prostaglandins and leukotrienes may participate in colon carcinogenesis and in tumor development (2-5).

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as sulindac and aspirin, are effective inhibitors of prostaglandin H synthase (PGH synthase) (6-8) and an inverse relationship between NSAID use and the relative risk of mortality due to colorectal cancer has been demonstrated (9-11), as well as reduction in tumor load in human familial adenomatous polyposis (FAP) patients (12-14) and in animal models with chemically-induced intestinal cancers (15, 16).

The C57BL/6J-Min (*Min/+*) mouse strain carries a dominant germline mutation in one allele of the tumor suppressor gene adenomatous polyposis coli (*Apc)* at nucleotide 2549 (17). This model is highly susceptible to spontaneous intestinal adenoma formation. The homozygous (*Min/Min*) mice are not viable, whereas the heterozygous (*Min/+*) mice develop over 30 adenomas throughout the entire intestinal tract (18). The *Min/+* mouse model is currently being used to study human intestinal tumorigenesis because it is genotypically and phenotypically analogous to the inherited human disease FAP (17, 19).

# PART II

# REVIEW OF THE LITERATURE

## INTESTINAL CANCER

Cancer is the second leading cause of death in the United States. About 25% of all deaths are due to cancer and is surpassed only by deaths due to heart disease which accounts for approximately 1/3 of the mortality. Colorectal cancer, next to lung cancer, represents the second leading cause of deaths from cancers in the Unites States. The colon is among the most common site for new cancer cases for both genders in the U.S. Colon cancer is only behind prostate and lung cancers in males and breast and lung cancers in females (1).

Colorectal cancer is common in Western societies, but rare in developing countries; however, a sharp increase in the incidence of colorectal cancer has been observed in Eastern Europe and Japan in the last decade. Comparative studies of the international incidence of colon cancer indicate that there is a more than 10-fold difference in incidence between the countries with the highest (the Japanese population in Hawaii for male and in New Zealand for female) and the countries with the lowest rate (in Africa and India for both genders) (20, 21), suggesting environmental factors may play an important role in incidence.

The origin of cancer development has been extensively studied during the past twenty years. Both genetic and environmental factors are considered as the responsible risk factors for human colon cancer (22). Most cancers of the colon occur as a result of a somatic mutation, and as such, sporadic colorectal cancer accounts for approximately 76% of colorectal cancer cases (23, 24). Some forms, however, are highly prevalent in families, where there appears to be a predisposition to the cancer as a result of a germline mutation.

In familial adenomatous polyposis (FAP), individuals develop large numbers of polyps in the colon. This form of hereditary colon cancer represents approximately 1% of colorectal cancer cases and is characterized by 100-1000s of intestinal adenomas, concentrated primarily in the colon. FAP patients develop colonic adenomas over a duration of 20-30 years. The polyps (adenomas) are not initially malignant; however, if left in place, they can eventually evolve into invasive carcinomas. A second form of hereditary colon cancer is known as hereditary nonpolyposis colon cancer (HNPCC) (accounts for about 5% of colorectal cancer cases). In contrast to FAP, there is no early polyp phase; however, once polyps form, they rapidly progress into carcinomas. Of all the risk factors associated with colorectal cancer, diet is among the most important environmental determinants in the etiology of this disease (25). High intakes of fat, saturated and polyunsaturated, are characteristic of the typical Western industrialized diet, and these fats are believed to exert a strong effect on the promotion and progression of colorectal tumors (25, 26).

## MOLECULAR GENETICS OF COLON CANCER

The development of human intestinal cancer involves progression through a series of distinct morphological stages and has long been thought to be a complex and multistep process (27-31). The progressive evolution of colon cancer develops slowly over years or even decades. With regard to genetic risk factors, several studies have revealed numbers of activated oncogenes, inactivated tumor suppressor genes and mismatch repair genes that appear to be involved in initiation, promotion, proliferation, and transformation (Table

2.1) (32). Recently, it has become possible to identify the molecular events that underlie the aberrant cell growth leading to colorectal cancer (33, 34). Analysis of specific genetic alterations at each of these morphological stages in humans has lead to the development of a model implicating an interplay of the accumulated mutations in several tumor suppressor genes and oncogenes in tumor formation and progression (Figure 2.1) (27, 29).

**Tumor suppressor genes and colon cancer**

Tumor suppressor genes, which generally have inhibitory functions to restrain cell growth, proliferation and malignant transformation, were first found in the mid 1980s and had drawn the attention of cancer research because they are commonly mutated in human cancer and the spectrum of mutations in these cancers provides clues to the etiology and molecular pathogenesis of neoplasia (28-29, 33). The accumulation of these genetic changes in tumor suppressor genes plays a key role in the transition from a benign adenoma into an invasive growing carcinoma (27, 29, 35). A mutated *p53* gene (named for its 53-kDa molecular weight gene product) has been found in DNA samples prepared from more than half of human tumors examined to date, making it the most common gene mutation associated with human cancer causation (36).

A loss of specific chromosomal regions occurs frequently in colorectal neoplasia. Usually the losses involve only one of the two parental chromosomes present in normal cells. These allelic losses have been interpreted as evidence that the regions affected contain tumor suppressor genes, whose products normally regulate growth and differentiation in a negative fashion and thus indirectly suppress neoplastic development (37). Loss of heterozygosity was most frequently observed in chromosomes 5q, 17p and

Table 2.1. Genetic risk factors related to colorectal tumorigenesis [§]

| Gene | Locus | Length (aa) |
|---|---|---|
| Oncogene | | |
| *K-ras* (Kirsten rat sarcoma) | 12p12.1 | 189 |
| Suppressor genes | | |
| *APC* (adenomatous polyposis coli — responsible gene) | 5q21 | 2843 |
| *p53* (protein 53 kDa Li-Fraumeni — responsible gene) | 17p12 | 393 |
| *DCC* (deleted in colorectal carcinoma) | 18q21.3 | 1447 |
| *MCC* (mutated in colorectal cancer) | 5q21 | 829 |
| DNA mismatch repairing genes | | |
| h*MSH*-2 | 2p16 | 1037 |
| h*MLH*-2 (All involved in HNPCC | 3p21 | 756 |
| h*PMS*-1 — responsible genes) | 2q31-33 | 932 |
| h*PMS*-2 | 7q22 | 862 |

[§] Modified from Ref. 32

aa: amino acid; p: short arm of chromosome; q: long arm of chromosome

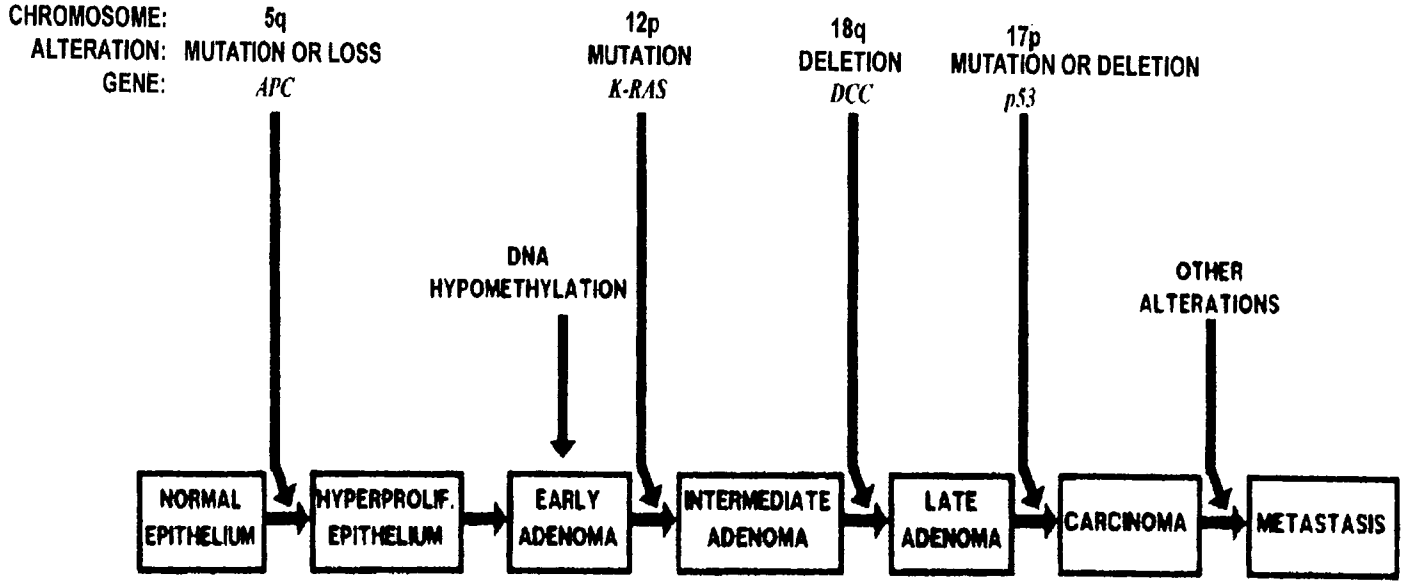


Figure 2.1. A genetic model of colorectal tumorigenesis. Colorectal tumorigenesis proceeds through a series of genetic alterations involving oncogenes and tumor suppressor genes. Mutation of the *APC* gene leads to abnormal epithelial proliferation and differentiation. An increase in the prevalence of *K-ras* mutation is evident in intermediate adenomas. Deletion of the *DCC* gene occurs more frequently in late adenomas. Deletion and mutation of the *p53* gene is associated with the development of carcinomas ( Modified from Ref. 27, 28).

18q, suggesting the presence of candidate suppressor genes in particular regions of these chromosomes. Studies directed at the identification of these genes led to the finding of the *p53* gene on chromosome 17p (38, 39), the *DCC* gene (deleted in colorectal cancer) on chromosome 18q (40-42), and the *MCC* gene (mutated in colorectal cancer) and *APC* gene (involved in adenomatous polyposis coli), both on chromosome 5q (43-46).

The current concept is that the *APC* gene has a gatekeeper function in the colorectum, i.e., this gene can be considered rate limiting for neoplastic growth and the dysplastic phenotype (47). The mutated *APC* gene appears to permit the outgrowth of early colonic polyps and therefore is thought to play a crucial role in tumor formation and progression. Recent advances in molecular genetics revealed that a germline mutation of *APC* gene is responsible for the inherited colon cancer associated with FAP. In addition, most sporadic cancers have a corresponding *APC* mutation and thus can occur randomly in colonic epithelial cells in most human colonic tumors. Later, a somatic mutation may activate a *ras* oncogene, creating a more advanced polyp that is, however, still benign. Over a period of years, this polyp may sustain mutations in its *DCC* and *p53* suppressor genes, and these mutations in turn appear to lead to the more autonomous, uncontrolled growth of the colon carcinoma cell (27).

**Tumor suppressor gene—adenomatous polyposis coli (*APC*)**

It is generally thought that genetic predisposition plays an important role in the induction of colon cancer in certain individuals. Current evidence suggests mutation of a gene on chromosome 5q is involved in the early stages of development of colorectal tumors. For example, allelic loss of a gene on 5q is noted in the smallest adenomas

examined, suggesting this is an early event, if not the initiating event, in colorectal tumorigenesis (47, 48). One particular type of genetic change that predisposes individuals to colon cancer is a mutation on the *APC* gene (43, 45, 46, 49). On the basis of genetic linkage analysis and subsequent chromosomal position cloning, the *APC* gene was mapped to human chromosome 5q21 (43, 45, 46, 49-52).

Alterations in the human *APC* gene have been demonstrated to play a role in familial adenomatous polyposis (FAP) as well as sporadic cancers (24). Germline mutations in the *APC* gene are known to be present in individuals with FAP, an autosomal dominant inherited predisposition to colorectal cancer (53). Somatic *APC* mutations can be found in over 70% of human sporadic colorectal tumors and this mutation occurs as an early event during the colorectal tumorigenesis as frequency of this mutation remains constant as tumors progress from benign to malignant stages (24). Recently, a mouse model system has been developed in which mice have a mutation mapped to chromosome 18 and is tightly linked to *Apc*, the mouse homolog of the human *APC* gene (54).

*APC* is a large gene consisting of 15 exons encoding a protein containing 2843-amino acid residues (Figure 2.2). Exons 1-14 are small, while exon 15 is large and two thirds of disease-causing mutations are found in the 5' (proximal) segment of this exon (55, 56). Antibodies to the APC protein have identified a 300 kDa cytoplasmic protein expressed in epithelial cells (57). This APC gene product contains an $NH_2$-terminal domain with potential to form a coiled-coil structure, a central region characterized by a repeated 15- to 20- amino acid sequence, and a cluster of basic amino acids in the COOH-terminal region (44, 45). The features of the APC protein have been well elucidated. The

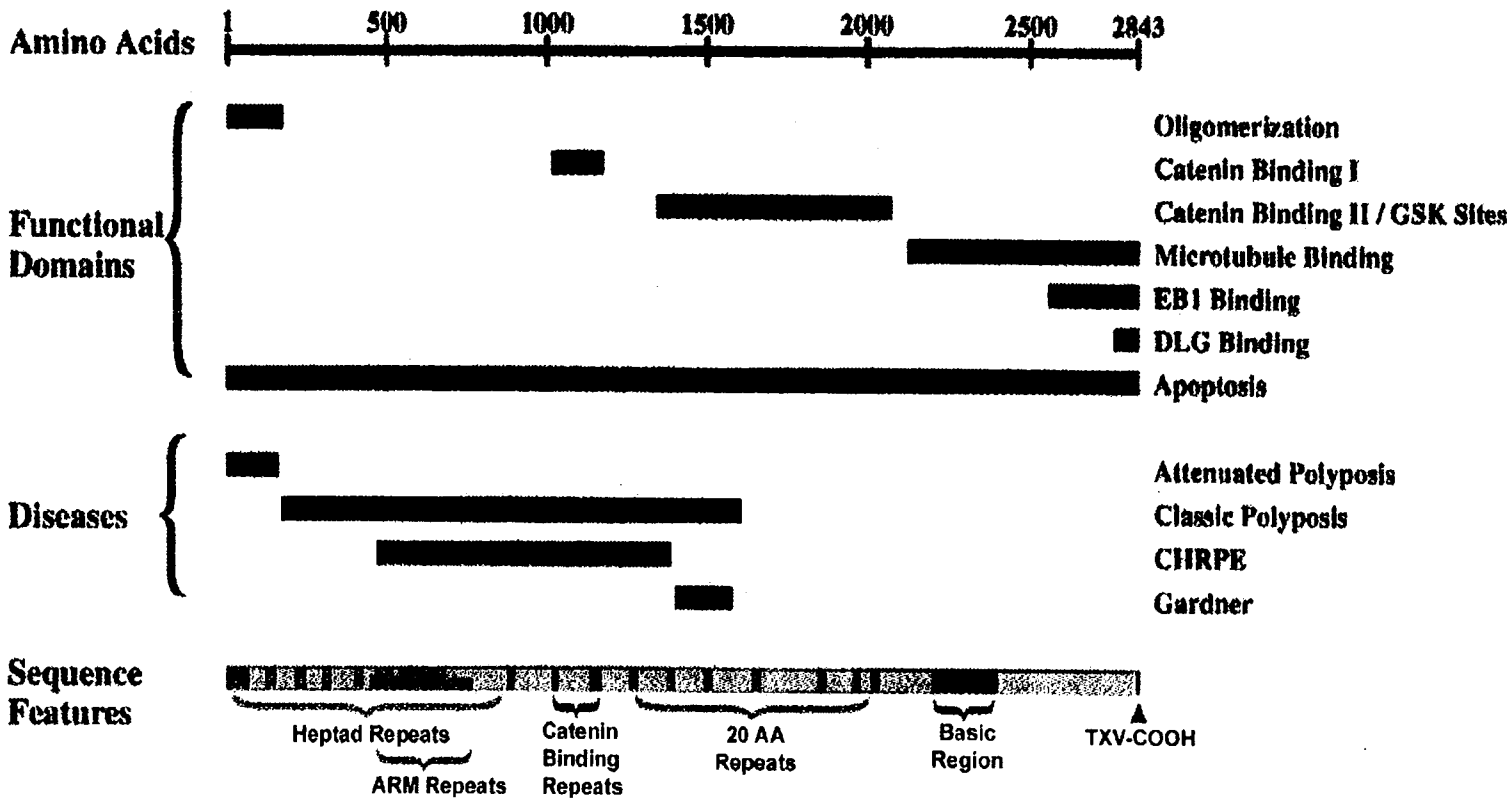


Figure 2.2. Functional, pathogenic properties and sequence features of *APC* (Reference 56)

extreme $NH_2$-terminal region is responsible for APC homodimerization. The first 45 amino acids of APC are necessary and the first 171 amino acids are sufficient for this interaction. Most mutant APC proteins still retain the capacity to associate with wild-type APC both *in vivo* and *in vitro* (58, 59). Recent studies strongly suggest that APC associates with alpha- and beta-catenins which are known to bind to the cell surface molecule E-cadherin. Some of the repeats in the central region of the protein appear to be responsible for this interaction (60, 61). Catenins are considered to be involved in the transmission of adhesion signals (62) and it is proposed that APC protein may affect the interaction between catenins and E-cadherin, thus influencing cellular adhesion and possibly intercellular communication (60, 61). Three distinct interactions have been described for the carboxyl terminus of APC. When overexpressed in colon cancer cell lines, the final 700 amino acids of APC can interact with microtubules (63, 64). Yeast two-hybrid analysis indicated that amino acids 2560-2843 of APC can bind a protein of unknown function called EB1 (65). Recently, two-hybrid studies have also shown that the final 72 amino acids of APC are sufficient for binding to the human homolog of *Drosophila* discs large tumor suppressor protein (DLG) (66). While the functional significance of these three carboxyl domains is unclear, binding of APC to microtubules and DLG may also regulate cell adhesion. It has been reported that a trimeric complex of DLG, APC, and β-catenin can be immunoprecipitated from mouse brain cells (66). The interaction of APC with microtubules has been hypothesized to mediate signals from β-catenin/cadherin to the cytoskeleton (63, 64).

While it remains unclear precisely how disruption of APC function can promote tumor formation, various possible mechanisms have been proposed. One of these hypotheses suggests that tumor formation is the result of haploinsufficiency, a threshold effect involving the deficiency of *APC* gene product (51). For example, the normal homozygote wild type produces enough of the *APC* gene product so that random fluctuations of its concentration never, or at least only very rarely, go below a threshold that permits localized excessive growth. The deficient heterozygote, however, may allow random fluctuations in the level of the *APC* gene product to go below this threshold relatively frequently. In those area of the colon where this happens, excessive epithelial growth may be initiated, giving rise to polyps. In a related hypothesis, *APC* is a recessive tumor suppressor gene and inactivation of both alleles is necessary for the cancer to develop. This is also known as the "two-hit" hypothesis (67, 68). That occurrence of somatic *APC* mutations in early adenomas from FAP patients supports this view (69, 70). In the other hypothesis, chain-terminating mutations in *APC* result in truncated polypeptides which can inhibit the normal function of the *APC* gene product in a dominant negative fashion. The great majority of *APC* mutations described to date result in stable >80 kDa truncated proteins, supporting this scenario (57). Further subcellular localization has demonstrated that wild type APC binds to microtubules and may be important in their formation (63, 64). However, mutant APC product (missing the carboxyl terminal part of the molecule as a result of premature truncation) appears to lack this ability (63). Other intriguing possibilities have been raised by the recent discovery that APC may be an important molecule in regulating cell adhesion. This hypothesis is based primarily on

evidence that APC can bind to and regulate β-catenin (60, 61, 71-73). β-catenin is one member of a family of intracellular catenins that regulate cell-cell adhesion between epithelial cells, in part through interactions with E-cadherin (74-76). Immunoprecipitation experiments have demonstrated that three imperfect 15 amino acids repeats located between residues 1020 and 1169 of APC are involved in a constitutive binding interaction with β-catenin in human cells (Figure 2.2) (60, 61). APC can trigger a post-translational down-regulation of β-catenin that appears to be mediated by binding of β-catenin to a second region of APC (71). This binding domain consists of several 20 amino acid repeats located between amino acids 1342 and 2075 of APC (60, 71). APC mediated regulation of β-catenin levels may be further controlled by activation of Wnt1 (the analogous molecule of Wingless (WG) in mice, where WG is a cell to cell signal in the fruit fly *Drosophila* that triggers many key developmental processes) and glycogen synthase kinase 3β (GSK 3β) (77, 78). These findings have led to a tentative model implicating *APC* in a Wnt1 signal transduction cascade (Figure 2.3) (72, 77, 78). In the absence of Wnt signals, APC simultaneously interacts with the serine kinase GSK 3β and β-catenin. Phosphorylation of APC by GSK 3β regulates the interaction of APC with β-catenin, which in turn may regulate the signaling function of β-catenin (78). Wnt signaling appears to antagonize GSK 3β activity. Upon Wnt signaling, β-catenin is stabilized and exists primarily as a cytoplasmic monomer (77). However, since *Wnt1* does not appear to be expressed in the intestine, it is unclear whether this pathway is fundamentally important in regulating intestinal tumorigenesis.

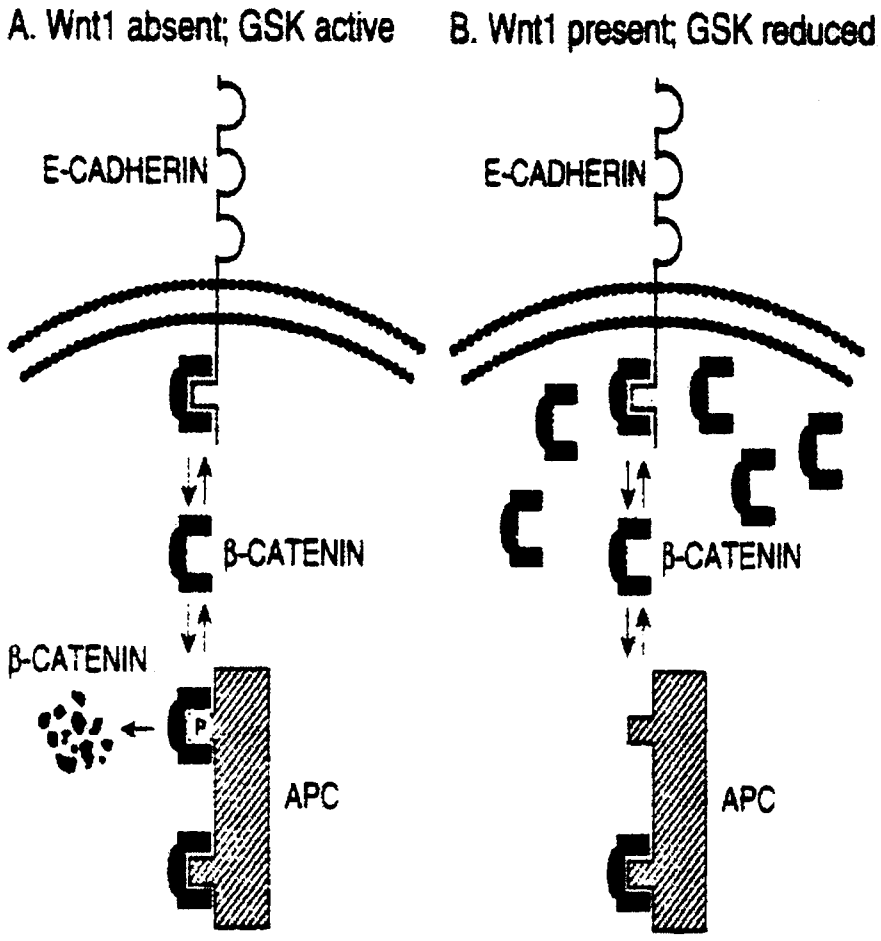


Figure 2.3. Potential role of APC in a Wnt1 signal transduction pathway. The interactions between APC, β-catenin, and E-cadherin as well as the potential regulation of these interactions by GSK 3β and Wnt1 are shown. In the absence of Wnt1, GSK 3β is postulated to be active. GSK 3β phosphorylation of APC enhances binding of β-catenin to the 20 aa repeats located between amino acids 1342 and 2075 of APC. Binding in this region of APC leads to β-catenin degradation. β-catenin also binds constitutively to the 15 aa repeats located between 1020 and 1169 of APC. Wnt1 activity is believed to reduce GSK 3β activity. Under these conditions β-catenin does not bind to the 20 aa repeats of APC, and therefore β-catenin degradation is prevented. Thus, intracellular levels of β-catenin increase. Note that β-catenin can still binds in the 15 aa repeat region of APC. (Ref. 79)

Taken together, the following summarizes the important features of the *APC* mutation:

1. Mutations of the *APC* gene are found in DNA isolated from sporadic tumors of digestive organs as well as in constitutional DNA of FAP patients.
2. Inactivation of both alleles of *APC* is required for development of most tumors in the colon and rectum.
3. The great majority of known mutations would result in truncation of the *APC* gene product.
4. Almost all known mutations are located within in the 5' (proximal) segment of the coding region in exon 15, although somatic mutations in colorectal tumors tend to cluster in the mutation cluster region (MCR) located between codons 1286 and 1513.
5. Most point mutations in *APC* are transitions from cytosine to other nucleotides.
6. The protein products of mutant of *APC* genes are defective in their ability to stimulate degradation and maintain low levels of cytoplasmic β-catenin.
7. Truncating mutations in amino terminus of APC are associated with a low penetrance in which patients develop a relatively small number of polyps.
8. Disruptions of APC between codons 1250 and 1464 or in carboxyl terminus region are associated with an increased number of colorectal tumors.

**Familial adenomatous polyposis (FAP)**

The original report of FAP is attributed to Cripps (80), who described the cases of a brother and sister whose rectums were thickly studded with 'several mulberry-looking masses of growth, varying in size from a small pea to a good sized nut'. Five years later, Smith (81) reported the presence of multiple polyps in three siblings and recognized the high risk of colorectal cancer associated with this condition. Subsequently, FAP was acknowledged to be transmitted as an autosomal dominant inherited condition with a high degree (>95%) of penetrance. Within the last decade, the molecular pathogenesis of FAP has been elucidated where the genetic linkage of FAP and *APC* has been located to a small region of the long arm of chromosome 5 (43, 45, 46, 49-52). Over 200 germline mutations of this very large *APC* gene have been described and most are predicted to result in a truncated protein that is dysfunctional in its role as a tumor suppressor.

It is well understood to date that familial adenomatous polyposis (FAP), in which affected individuals develop hundreds to thousands of colorectal adenomas (also called adenomatous polyps) during the second and third decades of life, is an autosomal dominant inherited human cancer disease that affects about 1 in 7000 individuals and is caused by a germline mutation of the *APC* gene (44-46, 51). These adenomas are not initially malignant, but if not removed, some are likely to give rise to adenocarcinoma. Indeed, adenomas have been suggested to be precancerous states for most colorectal tumors (82). Additionally, FAP patients often develop extracolonic manifestations, including retinal lesions, osteomas, desmoids of the skin, and brain tumors. The majority of the patients with FAP possess detectable mutations of the *APC* gene (24). The

detection of mutations is difficult because they usually involve small insertions, deletions, or changes in single base pairs, resulting in a truncated protein product (44). Genetic testing is now available for FAP through the use of DNA markers with a 99% accuracy. Unfortunately, this requires a previous specific diagnosis of FAP in two family members. Because each FAP family may have a different mutation, developing a screening test for individuals without a family history has proven difficult (83).

Another general principle illustrated by FAP concerns the complex relationship between genotype and phenotype. FAP patients do not develop uniform clinical features, despite the fact that all have mutations of the same gene and virtually all mutations result in C-terminal truncated proteins (56). In some cases, the difference in phenotype is due to the type of mutation. For example, retinal lesions (congenital hypertrophy of the retinal pigment epithelium, called CHRPE) are associated with truncating mutations between codons 463 and 1387 (Figure 2.2) (84). Truncating mutations N-terminal to codon 157 are associated with an attenuated form of FAP in which patients develop a relatively small number of polyps (Figure 2.2) (85). Some studies have suggested that mutations between codons 1250 and 1464 are associated with an increased number of colorectal tumors (53). On the other hand, patients with identical mutations can develop dissimilar clinical features. For example, some patients with identical truncating mutations develop features of Gardner's syndrome (mandibular osteomas and desmoid tumors) while others do not (46). The complex relationship between genotype and phenotype is also apparent in other hereditary cancer predisposition syndromes, including those associated with breast cancer (86). Whether these phenotypic difference result from environmental influences or

modifying genes is not known.

A compelling example of the relationship between phenotype and *APC* genotype is observed in *Min* mice. These mice develop multiple intestinal adenomas and have a truncating mutation of the murine *Apc* gene at a position similar to that found in many FAP patients (17).

***Min/+* mouse**

Contemporaneous with the search for the *APC* gene, a strain of mice containing multiple intestinal adenomas was developed following an ethylnitrosouria (ENU)-induced germline mutation of a C57BL/6J male mouse in 1990 (18, 87). Sequence analysis of the entire 8535 bp *Apc* cDNA identified that this model, the multiple intestinal neoplasia (MIN) mouse, has a fully penetrant dominant phenotype with a germline nonsense mutation (leu (TTG) → stop (TAG) transversion at nucleotide 2549, codon 850) in the murine homologue of the *APC* gene (*Apc*) (17). This mutation is analogous to those found in human FAP, in sporadic colorectal cancers and in hereditary nonpolyposis colon cancer (HNPCC) (23, 24, 88-90). Homozygous *Min/Min* mice are embryonic lethal, but heterozygotes (*Min/+*) on a C57BL/6J background mice develop numerous adenomatous polyps throughout the entire length of the intestinal tract within the first 3 months of life and rarely live past 150 days of age (18). Since all intestinal tumors in B6 Min/+ mice are benign adenomas, the premature death of these animals is associated with secondary effects of tumor growth, including morbidly chronic anemia (18).

The human and mouse *APC/Apc* genes are 86 and 90% identical at the nucleotide and amino acid levels, respectively (17). The *Min/+* mouse has a nonsense mutation at

codon 850 of *Apc*. The protein product of the *Apc* $^{Min}$ allele would contain the homodimerization domain as well as the central repeat region but would lack all other known APC binding domains. A comparison of the phenotypes associated with heterozygosity for germline mutations in *APC/Apc* for humans and Min mice is illustrated in Table 2.2.

The number of intestinal tumors and lifespan in *Min/+* mice were strongly affected by genetic background. For example, the *Min/+* $F_1$ progeny crossed with AKR strain (AKR/J x C57BL/6J) $F_1$ mice average 6.0±4.7 intestinal tumors per mouse and can live beyond 300 days. This change represents a significant decrease in tumor number from the average of approximately 30 tumors seen in *Min/+* mice derived from C57BL/6J background (18, 87). Similar results were found in $F_1$ progeny with two other mouse strains, MA and CAST (87). This finding suggested that the AKR, MA and CAST strains may carry genetic modifiers that act dominantly or semi-dominantly to reduce tumor number in *Min/+* mice. In 1993, linkage analysis demonstrated that a single locus, designed as *Mom-1* (for modifier of Min-1) in a region similar to a segment of human chromosome 1 that was mapped to a 15-cM region on mouse distal chromosome 4 accounts for much of this difference between strains (91). Thus, *Mom-1* seems to play an inhibitory role in the formation of polyps in the MIN mouse. Recently, MacPhee *et al.* (92) reported that a secretory phospholipase $A_2$ was an attractive candidate gene for the *Mom-1* locus. This gene *pla2s* is mapped to the same region (distal chromosome 4) that contains *Mom-1* and MIN mouse strains with higher *pla2s* expression develop fewer polyps.

Table 2.2. Comparison of phenotypes for mice and human with germline Apc / APC mutations[§]

| Mouse (*Min*/+) | Human |
|---|---|
| Established from an ENU-treated C57BL/6J male mouse and is associated with a defect in the *APC* gene resulted in a dominant nonsense mutation in the codon 850 of mouse *Apc* gene | Autosomal, dominant inherited disease |
| | Affects 1 in 7000 individuals |
| Mouse *Apc* gene mapped to chromosome 18 | *APC* gene mapped to chromosome 5q21 |
| Multiple colon adenomas | Multiple colon adenomas (100-1000s) |
| Small intestinal adenomas > 90% adenomas were found in small intestine (30-50 tumors/mouse) | Small intestinal adenomas |
| | Develop carcinomas from adenomas following a second mutation (i.e., *p53*, *k-ras*) |
| Cystic intestinal crypts | |
| Epidermoid cysts | Epidermoid cysts |
| Desmoid tumors | Desmoid tumors |
| Mammary adenoacanthoma | Gastric polyps |
| | Osteomasof skull and mandible |
| | Hypertrophy of retinal pigment epithelium |
| | Abnormal dentition |
| | Lipomas |

[§] Modified from Ref. 79

The *Min*/+ mouse model is currently being used in cancer research because of close similarities at the genotypic and phenotypic levels with human FAP. This was the first spontaneous murine intestinal tumor model and provides an excellent experimental tool for addressing many fundamental questions associated with intestinal neoplasia.

## ENVIRONMENTAL FACTORS IN COLON CARCINOGENESIS

Colorectal cancer is very common in North America, Western Europe, Australia, and New Zealand, whereas the age-standardized incidence rate of colorectal carcinoma is very low in developing countries such as India and Africa (20, 21). Observations on migrants to the United States from Japan, comparing cause-specific mortality rates in 1949 to 1952 with 1959 to 1962, show that the mortality rates of colon cancer among Japanese males have risen in one decade almost to equal the higher risks prevailing for United States caucasians (93). A similar trend was seen among Polish and Chinese immigrants to the United States (94) and among Polish and South East Asian immigrants to Australia (95).

Marked international differences in the incidence and mortality of colon cancer and increase of risk in populations migrating from low to high risk areas suggest that environmental factors, specifically dietary factors play an important role in the etiology of colon cancer. For example, the etiological studies indicate strongly that diets rich in fat, meat and animal proteins and low in fiber, fruits and vegetables are causally related to the risk of colon cancer. Gregor *et al.* demonstrated that colon cancer risk in populations was more strongly correlated with current diet than with the diet consumed 20 years

previously, suggesting that dietary fat/meat is more important at late promotional or progression stages than at the initiation stage (96). Similar studies of migrants showed that the risk of colorectal cancer was correlated with the diet of the current residence rather than the place of birth.

**Dietary fat and colon cancer**

Dietary fat has received considerable attention as a risk factor in the etiology of colon cancer (4, 97, 98). Several animal model studies have provided evidence that the fatty acid composition of dietary fat is one of the determining factors in colon carcinogenesis in which fatty acids are thought to play an important role in both the development and prevention of colorectal cancer (97, 99-102). Saturated and n-6 polyunsaturated fatty acids promote colon cancer in a laboratory animal model (103-109). On the other hand, accumulating evidence suggests that n-3 PUFAs, particular eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), found in fish oil, inhibit cancer formation (4, 107-115). Consumption of fish appears to protect against colon cancer in human (116-118). Although the mechanism(s) of colon tumor-promoting/suppressing effect of dietary fatty acids is not precisely understood, the modulation of colon tumors by dietary fat is thought to be mediated, in part, through alterations of membrane phospholipids and eicosanoid biosynthesis (4, 109).

**n-3 polyunsaturated fatty acids**

Marine fish oils are rich sources of unsaturated fatty acids differing in chemical structure from comparable polyunsaturated lipids in plants. Two of the most well known marine fatty acids are EPA and DHA. Chemists have called the polyunsaturated fatty

acids in fish oil n-3 (or omega-3) fatty acids, because they have a characteristic double bond 3 carbons from the methyl end of the molecule. Structurally they differ from n-6 polyunsaturated fatty acids found predominantly in plant oils because the first double bond in these fatty acids is located 6 carbons from the methyl end of the molecule (119).

The biological properties of fish oils center around their ability to reduce the synthesis of prostaglandins and leukotrienes generated from the metabolism of arachidonic acid, arguably the most important n-6 polyunsaturated fatty acid associated with membrane phospholipids (120). Fish oils, by suppressing the formation of these agents, have anti-inflammatory properties and have been investigated in animals for cancer-preventive effects. In tumorigenesis studies, fish oil diets reduce the incidence of chemically-induced focal areas of dysplasia and colonic tumors in rodents (111, 112, 121). In a study with spontaneous intestinal tumor model, *Min*/+ mice, Paulsen *et al.* demonstrated that long-term (up to 18 weeks) dietary treatment with K85, a fish oil derived concentrate enriched in EPA and DHA, suppressed the formation and growth of small intestinal tumors (122). This finding is similar to the antitumor effect of dietary DHA in the $Apc^{\Delta 716}$ knockout mouse, a model characterized by 100s of intestinal tumors (123).

The inhibition of prostaglandin release may explain in part the beneficial effect of fish oil in controlling proliferation (112, 124). Prostaglandins appear to enhance carcinogenesis (125) and modulate proliferative activity in rat colonic epithelium (126), although their major effect may not be on proliferation per se but rather to slow migration and retard senescence (127, 128), thus prolonging cell life and delaying loss of epithelial

cells. Inhibition of prostaglandins by fish oil could accelerate cell loss, thereby reducing exposure of senescent cells to carcinogenic transformation.

**n-6 polyunsaturated fatty acids**

Linoleic acid (LA, 18:2 n-6) is the most commonly consumed PUFA in the American diet and is the parent compound (metabolic precursor) of the n-6 family of PUFA that includes arachidonic acid (AA). The origin of tissue AA is LA when LA is the only n-6 PUFA in the diet. A number of cancers, including those of the intestines, have been strongly linked to the amount of LA in the diet (97, 129-131). Increasing the amount of LA in the diet of rodents enhances the growth of chemically-induced colon, breast and pancreatic tumors (97, 132-134). The mechanisms responsible for LA's effects are uncertain, but because LA is the biological precursor of AA and eicosanoids, a commonly proposed hypothesis suggests that LA mediates its effects through AA. AA content in colonic mucosal phospholipids is associated with higher indices of cell proliferation (135) and tissue AA content is significantly correlated with eicosanoid biosynthesis (136). However, despite these relationships the mechanism of action by LA is still unclear because its conversion to AA and subsequently to eicosanoids is highly regulated (137). In fact, if the diet already contains sufficient levels of LA (i.e., 3% of energy), increasing consumption has little effect on either tissue AA content or eicosanoid biosynthesis (137).

**Eicosanoids**

Eicosanoids are oxygenated derivatives of 20-carbon polyunsaturated fatty acids. Among these, arachidonic acid is the preferred precursor from which most metabolic pathways have been described. Arachidonic acid is released from the sn-2 position of

membrane phospholipids by phospholipases, $A_2$, C and D mediated pathways (138). Of the various phospholipases, $PLA_2$s which directly cleave fatty acids from the sn-2 position are considered the most important (139). Once arachidonic acid is released, it is further metabolized to eicosanoids via one of three pathways. Eicosanoids are biologically active regulators with a wide range of physiological functions such as lymphocyte and platelet activation/aggregation, vasodilation, vasoconstriction, regulation of blood pressure, blood clotting, the immune system and modulation of the reproductive system to name a few. They are also involved in pathological processes such as inflammation, allergies and certain cancers (140-142). Eicosanoid production can be regulated by dietary fats. For example, animals consuming the n-3 PUFA produce less prostaglandins, while low to moderate levels of arachidonic acid (AA, 20:4n-6) augment eicosanoid production (136, 137, 143-146).

The eicosanoids, consisting of prostaglandins and thromboxanes, are one set of bioactive products of arachidonic acid metabolism. Arachidonic acid can be metabolized via three major pathways: a) the epoxygenase pathway produces epoxy and dihydroxy acids, b) the lipoxygenase pathway produces leukotrienes, lipoxens and hydroxy acids, and c) the cyclooxygenase pathway results in prostaglandins and thromboxanes (138). Collectively all of these oxidized products are known as eicosanoids. They are biologically active compounds, each with a diverse set of highly specific physiologic actions, some of which are in opposition to the actions of other eicosanoids. For example, prostaglandin $E_2$ ($PGE_2$) has vasodilatory and bronchodilatory properties while the leukotrienes, $LTC_4$, $LTD_4$, and $LTE_4$ have broncoconstrictive and vasoconstrictive

properties (147). These are products from different pathways usually in different cells and a balance is necessary for the homeostasis of airway openings and of vascular tone. Thromboxane $A_2$ ($TXA_2$) produced by platelets stimulates their aggregation while prostacyclin ($PGI_2$) produced by the endothelial cells, inhibits this aggregation (147). These products, both from the cyclooxygenase pathway, are produced by different cells and help maintain a coagulation balance in the blood. It is evident that the achievement of the necessary balance for homeostatic function requires that these molecules and their functions must be carefully regulated. This regulation is achieved by the cells responding to highly specific stimuli which arise from cell-cell interactions, receptor-mediated contact with secreted soluble molecules, or even physical stresses such hypoxia or shearing forces (138).

There are two key regulatory points for eicosanoid synthesis (148). The first is the liberation of free arachidonic acid from the glycerylphospholipids in cellular membranes. Major donors for arachidonic acid formation include phosphatidylinositol and phosphatidylcholine. Phosphatidylinositol can be cleaved to liberate arachidonate either by the sequential actions of phospholipase C, diacylglycerol lipase, and monoacylglycerol lipase, or directly by the action of phospholipase $A_2$. Arachidonic acid is cleaved from phosphatidylcholine via phospholipase $A_2$. Quantitatively, $PLA_2$ action is considered to be the more significant contributor of arachidonic acid release in most cell types. This step is considered the rate limiting step for prostaglandin formation (149).

The second point of regulation is the first committed step of prostanoid production, the conversion of arachidonic acid into $PGH_2$ by prostaglandin G/H synthase

(PGHS), commonly known as cyclooxygenase. This reaction encompasses two steps, first a bis-oxygenase activity catalyses conversion of arachidonic acid to $PGG_2$ then a peroxidase activity reduces the 15-hydroperoxyl group of $PGG_2$ producing $PGH_2$. Both the bis-oxygenase (or cyclooxygenase, hence the PGHS common name) and peroxidase activities are accomplished by the same protein that has two different, albeit interacting active sites (150, 151). The ability to separate these two activities is demonstrated by a phenylalanine substitution of Tyr 385 which eliminates the oxygenase activity but not the peroxidase activity (152) and by aspirin inhibition which eliminates cyclooxygenase activity but not the peroxidase activity (153). In addition, PGHS is considered a suicide enzyme since it inactivates itself during catalysis (154). Details of the enzymatic reactions are reviewed by Smith, Marnett, and DeWitt (138). PGHS is an integral glycoprotein with an associated heme group required for both activities (155). It has been localized to the endoplasmic reticulum and nuclear membrane (156) and appears to function as a homodimer (151).

The last step in the production of the variety of prostaglandins and thromboxanes in a given cell is accomplished by the rapid isomerization of $PGH_2$ by a panel of isomerases specific for a given cell type yielding $PGD_2$, $PGE_2$, $PGF_2$, $PGI_2$ or $TXA_2$ depending on the cell type (148). $PGH_2$ is very unstable and will nonenzymatically convert to the PGE series if not acted on enzymatically (157). The profile of eicosanoid products produced reflects the complement of these isomerases present in a given cell type. However, the amount of product produced reflects both the availability of arachidonate as substrate and the amount of active PGHS. Beyond providing cell product

specificity, there is little evidence suggesting the isomerases are significant regulatory points in eicosanoid synthesis. Albeit, there are reports in endothelial cells and murine 3T3 cells that PGI synthase can be co-regulated with PGHS (158, 159).

**Eicosanoids and cancer**

The role of eicosanoids in the development of colon cancer and their regulation of tumor growth and metabolism has received much attention. The evidence that eicosanoids may be involved in the pathogenesis of colon cancer is mainly based on the following two sets of findings. First, the metabolism of arachidonic acid is altered in human colon cancer, carcinogen-induced animal models and cancer cell lines. For example, the prostaglandin levels are higher in tumor cell lines (160) and in intestinal tumors compared to normal mucosa (161), and the activity of cyclooxygenase (the rate limiting enzyme for eicosanoid biosynthesis) is enhanced during colonic carcinogenesis (162-164). Venous blood draining colon tumors contains large amounts of $PGE_2$; the larger the tumor the higher $PGE_2$ output (165). The second group of findings results from the ability of NSAIDs to protect experimental animals and humans from colon cancer (3, 12, 166-170). Since NSAIDs are the best known inhibitors of PG synthesis, the commonly proposed hypothesis is that their effect on colon cancer is brought about via inhibition of PG synthesis (171), and that, in turn, PGs are crucial in the pathogenesis of colon cancer.

## PROSTAGLANDIN H SYNTHASE (PGHS; CYCLOOXYGENASE, COX)

Prostaglandin H synthase (PGHS), which is also referred to as cyclooxygenase (COX) is considered a key enzyme physiologically because it catalyzes the oxygenation

and endoperoxide reduction reactions required for the biosynthesis of prostaglandins and thromboxanes from arachidonic acid (148). These potent biological signaling molecules mediated a multitude of diverse biological functions such as inflammation, immune response, platelet functions, blood pressure, reproduction and cell proliferation or differentiation (172). Each of these functions may be regulated through several pathways, collaboratively mediated by prostaglandins.

PGHS is a dimeric enzyme consisting of identical 70-72 kDa subunits (148). PGHS is an integral membrane glycoprotein found primarily in the intraluminal compartment of endoplasmic reticulum and nuclear envelop. It contains two catalytic activities; a cyclooxygenase activity that adds two molecules of $O_2$ to arachidonic acid (AA), forming the hydroperoxy arachidonate, $PGG_2$; and a peroxidase activity that reduces $PGG_2$ to the endoperoxide, $PGH_2$ (148, 172). Each 70 kDa subunit requires one molecule of bound heme for maximal activities of both kinds (173). The cyclooxygenase and peroxidase active sites are separate on the protein molecule, thus the effect of aspirin and other NSAIDs are modulated through the inhibition of cyclooxygenase activity but occur without affecting peroxidase (174, 175).

Two forms of cyclooxygenase are known to be present in eukaryotic organisms: a COX-1 first purified from ram seminal vesicles encoded by a 2.8-kilobase mRNA, and a newly discovered mitogen-inducible COX-2 encoded by a 4-kilobase mRNA (176). COX-1 was initially characterized, purified and cloned from sheep seminal vesicles (150, 177, 178) and is 90% homologous at the amino acid sequence level in murine, rat and human (139). COX-1 is expressed constitutively in almost all cell lines and virtually all

mammalian tissues and can therefore produce prostaglandins in immediate response to hormones. (148, 179). Tissues that express high levels of COX-1 include platelets, endothelial cells, stomach, kidney, and smooth muscles (138, 148). COX-1 is believed to be responsible for normal cellular homeostasis or "housekeeping functions" such as platelet aggregation, anti-aggregation by endothelial cells, blood pressure regulation (172). In 1989, Simmons *et al.* (180) identified a second, inducible form of cyclooxygenase, known as prostaglandin endoperoxide synthase-2 (COX-2). This cyclooxygenase isoform was independently identified by differential screening of a phorbol ester-stimulated Swiss-3T3 fibroblast cDNA library (181). Unlike COX-1, COX-2 is virtually undetectable in most cell types under normal physiological conditions, but its expression can be rapidly induced by a wide range of extracellular and intracellular stimuli, including lipopolysaccharide (176, 182, 183), forskolin (184), interleukin-1 (IL-1), tumor necrosis factor (TNF) (185, 186), serum (187, 188), epidermal growth factor (EGF) (189), transforming growth factor-α (TGF-α) (190), platelet activating factor, retinoic acid (191), and endothelin (192). Additionally, the formation of COX-2 protein parallels the increase in eicosanoid production that is commonly the result of mitogenic stimulation in a wide variety of cell types. For example, prostaglandin production increases rapidly in rat intestinal epithelial cells (RIE) after stimulation with mitogens such as TGF-α or EGF (190). In resting unstimulated macrophages, only COX-1 but not COX-2 was detected (176), hence prostaglandins are not being produced by this enzyme (COX-2).

The genomic structure of both human and murine COX-1 is composed of 11 exons and 10 introns spanning 22.5 kb (Figure 2.4) (193-195). The human COX-1 locus maps

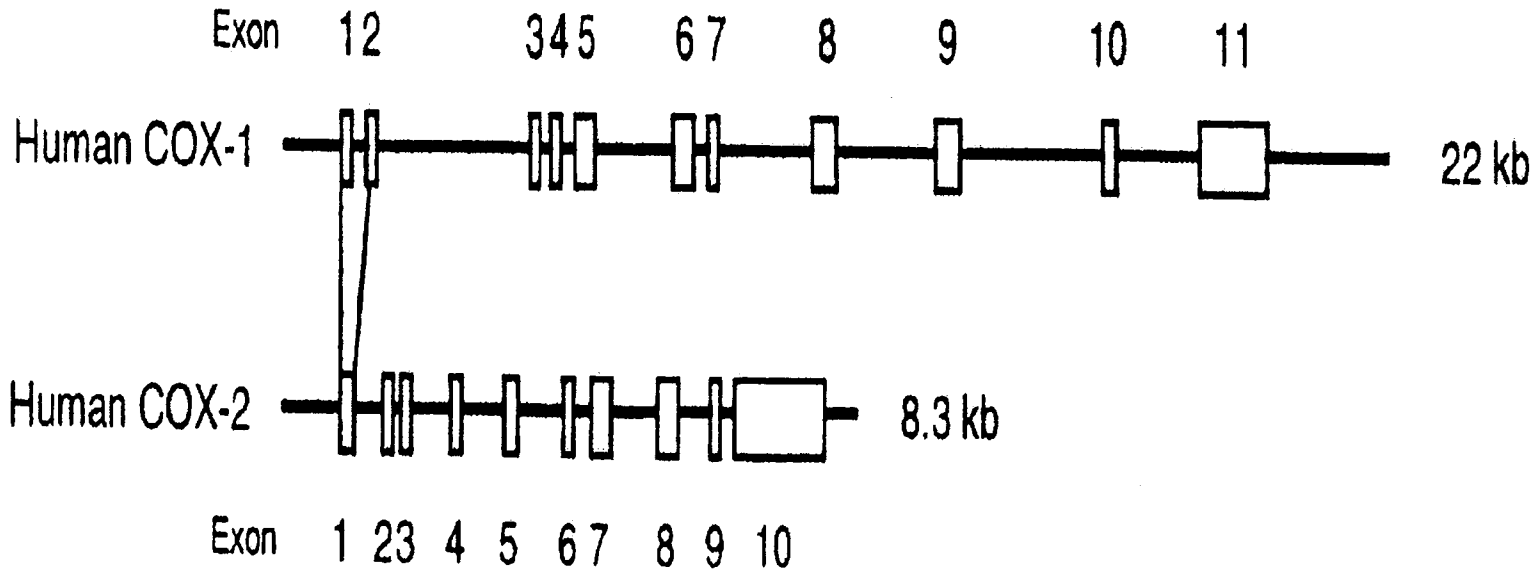


Figure 2.4. Gene structure of human COX-1 and COX-2 (Ref. 195)

to chromosome 9q32-q33.3 (193). Although COX-1 and COX-2 proteins are similar in size (70-72 kDa), the human COX-2 gene is only 8.3 kb in size. This reduction in size is primarily due to smaller introns as well as exon 2 is absence in COX-2 (Figure 2.4). Fluorescence in situ hybridization localized human COX-2 to chromosome 1q25.2-q25.3 (196).

COX-1 mRNA encodes a 565-residue, 70 kDa protein containing a short signal peptide and four possible N-linked glycosylation sites (Figure 2.5). COX-2, a roughly 70 kDa protein, is ~ 75% homologous to the COX-1 protein. At the protein level, major domains such as the serine acetylation site, the cyclooxygenase and peroxidase regions are conserved between the two isoforms; however, there are some other differences in the primary amino acid sequence. COX-1 contains a 26 amino acid leader sequence in the amino terminus while COX-2 contains a shorter, nonhomologous 17 amino acid leader sequence (Figure 2.5) (197). COX-2 possesses an 18 residue insert at the carboxyl terminus that is absent from COX-1 (139, 184). It is possible that these subtle differences in the primary amino acid sequence may cause differences in the secondary and tertiary structure of the protein that could affect the active site of these two enzymes. For example, COX-1 is completely inhibited by aspirin treatment, whereas COX-2 converts arachidonic acid to 15-*R*-HETE after aspirin treatment, functioning as a 15-lipoxygenase (198, 199). In addition, these differences at the $NH_2$ terminal and COOH terminal of these isoforms may affect their subcellular localization or stabilities of the two proteins. Each protein is localized predominantly in the endoplasmic reticulum (ER) and nuclear envelope (NE) when examined by immunocytofluorescence using isozyme specific antibodies (200).

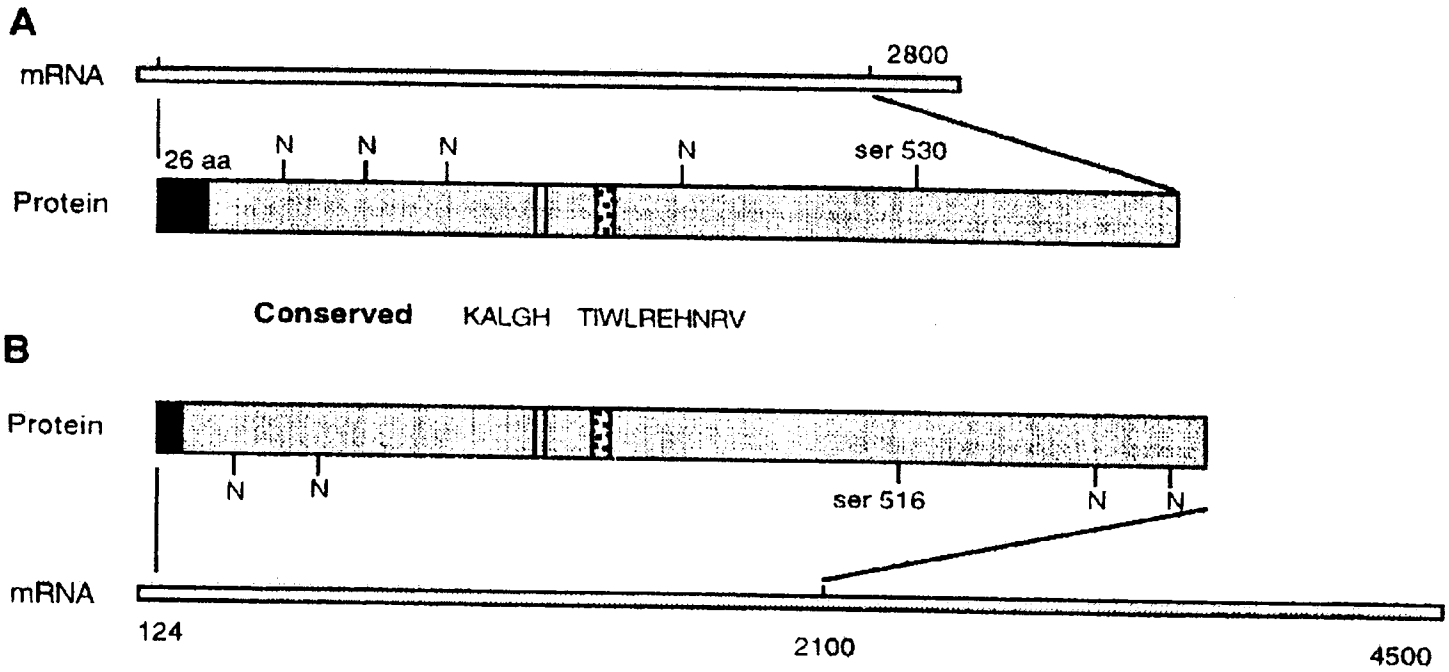


Figure 2.5. Comparison of cDNA and protein sequences for COX-1 and COX-2. Ser-530 (COX-1) and Ser-516 (COX-2) are aspirin acetylation targets. N, N-linked glycosylation; aa, amino acid residue; ser, serine. Open bars represent linear structure of cDNAs for COX-1 (A) and COX-2 (B). Shaded bars in middle represent linear amino acid sequence for COX-1 (A) and COX-2 (B). Each enzyme contains 4 N-linked glycosylation sites and regions of amino acid identify that are marked by amino acid sequence provided in middle. (Reference from Williams and DuBois, 1996)

COX-1 immunoreactivity was equally distributed between the ER and NE whereas COX-2 immunoreactivity was twice as concentrated in the NE as in the ER. Additionally, histochemical staining for enzyme activity has shown that COX-1 functions predominantly in the ER whereas COX-2 is active in both the ER and NE (201). These differing subcellular locations support the emerging view that COX-1 and COX-2 represent two independently operating eicosanoid biosynthesis systems. COX-1 produces prostaglandins that mediate housekeeping functions while eicosanoid products produced via COX-2 could mediate inflammation and could be preferentially distributed to the nuclear compartment of the cell and therefore may modulate intranuclear differentiation / proliferative functions and transcription of target genes.

**COX-1 and COX-2 knockout mice**

Gene disruption techniques have recently been utilized to understand the biological relevance of a variety of regulatory molecules including enzymes, cytokines, growth factors, transcription factors, and cell surface receptors. Important to COX research was the successful generation (by targeted gene disruption) of COX-1 and COX-2 deficient mice (202-204). COX-1 knock out mice survive as well as wild type animals, have no gastric pathology, and show less indomethacin-induced gastric ulceration than wild type mice. They also show reduced platelet aggregation and a decreased inflammatory response to arachidonic acid, but not to phorbol ester stimulation.

COX-2 null mice show no inbred gastrointestinal pathology, but their kidneys exhibit progressive deterioration during aging. Their inflammatory responses to phorbol ester and arachidonic acid do not differ from wild type mice and they have a normal

inflammatory response to bacterial invasion of the peritoneum.

These sets of observations suggest the need to evaluate the current view concerning the detailed functions of COX isoforms, for example, that prostaglandins produced from COX-1 protect against stomach ulceration and that the inflammatory response is due to prostaglandins produced by COX-2. Clearly, the knockout mice will continue to be of great value for the study of the precise roles of the two isoforms of COX and to aid in the development of novel NSAIDs.

**Role of cyclooxygenase in cancer**

A role for cyclooxygenase in carcinogenesis has been demonstrated in epidemiological studies in which mortality from colorectal cancer was reduced approximately 40-50% in individuals taking aspirin (9, 11, 205). In a related finding, sulindac treatment reduced the size and number of rectal polyps in patients with FAP (13). When sulindac administration was discontinued, the tumors recurred. If treatment was resumed, the tumors regressed again (206). In rat and mouse model of colon carcinogenesis, NSAIDs including indomethacin, piroxicam and sulindac have had chemopreventive effect as judged by a reduction in the number of tumors per animal (15, 171, 207). The apparent mechanism for the clinical and experimental effects of NSAIDs is inhibition of COX and subsequent reduction in the levels of prostaglandins. The molecular basis for the beneficial effects of NSAIDs in cancer is little understood and complicated by the multistage nature of the disease.

The relative contributions of the COX-1 and COX-2 isozymes to carcinogenesis is not known and has only recently become an intense area of investigation. COX-2 appears

to be the isoform responsible for elevated prostaglandin production in neoplastic cells. The mRNA of COX-2 but not COX-1 was markedly elevated in colorectal adenocarcinomas and a subgroup of colonic adenomas as compared with levels in adjacent normal tissues, this may indicate that increased expression of COX-2 is involved in the progression to a transformed state (208). Enhanced COX-2 expression was also observed in patients clinically diagnosed with colorectal cancer (208-210). In mouse skin tumor models, COX-2 protein was constitutively overexpressed in carcinomas (211). On the other hand, COX-1 expression remained comparable in tumors and normal appearing tissue in those studies. In general, the extent and intensity of immunoreactive COX-2 in cancer cells is much greater than that of other cell types. In contrast, the expression of COX-1 is weak in both normal and cancerous specimens. These data suggest that increased prostaglandin production in colon cancer tissues may be due to enhanced expression of COX-2. Furthermore, selective inhibition of COX-2 may prove to be more efficacious in the retardation of colon cancer cell development. These observation also suggest that beneficial effect of NSAID treatment against colon cancer may be mediated by inhibition of COX-2. In addition, COX-2 is found predominantly in the nuclear envelope and COX-1 in ER, suggesting that the two enzymes represent separate signaling systems. The nuclear localization of COX-2 and its inducibility in response to mitogenic stimuli link it to proliferation.

## NONSTEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are common anti-inflammatory

therapeutic agents. In 1986, more than 100 million prescriptions were written for these drugs (212). During the past 30 years, there has been a substantial increase the number of NSAIDs. In the United Sates, salicylates were the most common treatment for rheumatic disease (213) and 14 NSAIDs are now available. As a group, NSAIDs have anti-inflammatory, analgestic, antipyretic and platelet-inhibitory actions, though they certainly do differ chemically and pharmacokinetically.

NSAIDs currently available come from a variety of chemical classes (Table 2.3). Their physicochemical properties determine their distribution in the body, and thus differences in these properties may lead to variable therapeutic performance (214).

NSAIDs are a family of pharmacological compounds whose major mechanism of action is inhibition of cyclooxygenase (6). Since 1991, several reports indicate an association between an intake of NSAIDs and a decreased risk for developing colorectal cancer. Three independent lines of research indicate an inverse correlation between NSAIDs use and colorectal carcinogenesis. First, most epidemiological studies show a 40-50% reduction in mortality and the relative risk of colorectal cancer in individuals who regularly consume NSAIDs (like aspirin) compared with those not taking these agents. Second, the NSAID sulindac has been shown to significantly reduce the number and size of adenomatous polyps in patients with familial adenomatous polyposis (FAP). Third, in line with the findings in humans, animal models of colorectal carcinogenesis studies have demonstrated that several different NSAIDs provide chemoprotective effects against colon carcinogenesis as shown by a reduction in the tumor load and size.

Table 2.3. Chemical classification of NSAIDs[§]

| | |
|---|---|
| Carboxylic acids | |
| Acetylated | aspirin* |
| Nonacetylated | choline salicylate, diflunisal, magnesium salicylate, salicylamide, salicylate with magnesium salicylate, salsalate, and sodium salicylate |
| Acetic acids | diclofenac*, indomethacin*, tolmetin*, sulindac*, and etodolac* |
| Propionic acids | ibuprofen*, naproxen*, fenoprofen*, pirprofen, indoprofen, tiaprofenic acid, oxaprozin, ketoprofen*, fenbufen, flurbiprofen, carprofen, and suprofen |
| Fenamic acids | flufenamic acid, mefenamic acid*, meclofenamic acid*, and niflumic acid |
| Enolic acids | oxyphenbutazone, phenylbutazone*, piroxicam*, sudoxicam, tenoxicam, and isoxicam |
| Nonacidic compounds | nabumetone, proquazone, and bufexamac |

[§] Modified from Ref. 214

* Available in the United States

**Epidemiological studies of NSAIDs and colorectal cancer**

There have been a number of observational retrospective and case control studies on the relationship of intake of aspirin or other NSAIDs and the subsequent risk of developing colorectal cancer (9, 11, 205, 215-221). These studies are summarized in Table 2.4. All of these studies have been consistent and demonstrated a protective effect of NSAIDs; an approximately 50% decreased risk of colorectal cancer is noted in groups taking NSAIDs, primarily aspirin.

Many but not all prospective cohort studies examining the effect of aspirin on the incidence or mortality of colon cancer indicated a protective effect (Table 2.5) (11, 220-224). Thun and colleagues (11) reported the results of the largest and most frequently cited study of the relationship between aspirin use and colon cancer. They conducted a cohort of over a million volunteers and family members with a questionnaire on cancer risk and personal health habits, including medication use. Complete information was available for over 660,000 persons for the 6-year study period. Death rates from colorectal cancer decreased with more frequent aspirin use. The relative risk for fatal colorectal cancer among persons using aspirin every other day or more a month was 0.60 in men and 0.58 in women. In a subsequent report, Thun and associates evaluated aspirin use and the risk of any fatal cancers (223). Mortality rate decreased with more frequent aspirin use for cancers of the oesophagus, stomach, colon and rectum. On the other hand, some studies suggested that the use of aspirin, at a dose adequate to prevent myocardial infarction, was not associated with protection against colorectal cancer (225, 226).

Table 2.4. Summary of retrospective case control studies of NSAIDs use and colorectal cancer incidence

| Authors | Setting | Study Size | End Point | NSAID Dose | Results Relative Risk | Comments |
|---|---|---|---|---|---|---|
| Kune *et al.* (1988) | Population based | 715 cases<br>727 controls | Colorectal cancer (incidence) | Aspirin daily<br>NSAIDs daily | 0.60 (0.40-0.82)<br>0.77 (0.60-1.01) | Adjusted for age, sex, diet and physical activity (community controls) |
| Rosenberg *et al.* (1991) | Hospital based | 1326 cases<br>4891 controls | Colorectal cancer (incidence) | Aspirin ≥4 days/week ≥ 3 months | 0.5 (0.40-0.80) | Adjusted for age, sex, race, religion, physical activity, family history, and educational level (cancer and noncancer controls) |
| Logan *et al.* (1993) | Community based | 147 cases<br>153 controls | Colorectal adenomas | NSAIDs any use | 0.49 (0.3-0.8)<br>0.66 (0.4-1.1) | vs. negative controls for fecal blood<br>vs. positive controls for occult blood |

Table 2.4. (Continued)

| Authors | Setting | Study Size | End Point | NSAID Dose | Results Relative Risk | Comments |
|---|---|---|---|---|---|---|
| Suh *et al.* (1993) | Hospital based | 830 cases<br>212 cases Polyps<br>1662 controls | Colorectal cancer | Aspirin<br>< 1 day/week<br>1-2 days/week<br>> 2 days/week | <br>0.83 (0.43-1.61)<br>0.49 (0.24-0.99)<br>0.44 (0.18-1.10) | Adjusted for age, sex, educational level, and duration of use |
| Peleg *et al.* (1994) | Hospital based | 97 cases<br>338 controls | Colorectal cancer (incidence) | Aspirin<br>< 1 day/week over 4 years<br>> 3 days/week over 4 years | <br>0.52 (0.30-0.91)<br>0.08 (0.01-0.59) | Use during 4 years before study |
| Muscat *et al.* (1994) | Hospital based | 511 cases<br>500 controls | Colorectal cancer (incidence) | NSAIDs<br>≥ 3 days/week for ≥ 1 year | <br>0.64 (0.42-0.97)<br>0.32 (0.18-0.57) | <br>Men<br>Women |

RR = relative risk (incidence rate among exposed/incidence rate among unexposed)

Table 2.5. Summary of prospective studies and clinical trials of NSAIDs use and colorectal cancer

| Authors | Setting | Study Size | End Point | NSAID Dose | Results Relative Risk | Comments |
|---|---|---|---|---|---|---|
| Paganini-Hill *et al.* (1989) | Cohort: LA retirement community 1981-1988 | 13,897 181 cases | Colon cancer (incidence) | Aspirin daily | 1.5 (1.1-2.2) | Elderly (median age 71) Aspirin use determined only on initial interview; No ASA data after baseline |
| Thun *et al.* (1991) | Cohort: American Cancer Society 1982-1988 | 662,424 950 cases | Colon cancer (fatal) | Aspirin < 1 use/month | For men 0.77 (0.61-0.97) | Aspirin use determined only on initial in Results for women were similar. |
| | | 138 cases | Rectum cancer (fatal) | 1-15 use/month | 0.69 (0.52-0.93) | |
| | | | | ≥ 16 use/month | 0.60 (0.40-0.89) | Decreased risk mostly confined to users of ≥ 10 yrs |
| Greenberg *et al.* (1993) | U.S. polyp prevention trial | 793 patients with adenomas | Recurrent colorectal adenomas | Aspirin any use at start and end of 1 year | 0.52 (0.30-0.89) | Colonoscopy minimized detection bias |
| Gann *et al.* (1993) | U.S. Physicians's Health Study 1982-1988 | 22,071 men 33 cases | Colorectal cancer | Aspirin 325 mg every other day | 1.15 (0.80-1.65) | The only available randomized trial but short follow up. This study was terminated after 4 years |
| | | | Colorectal polyps | | 0.86 (0.68-1.10) | |

Table 2.5. (Continued)

| Authors | Setting | Study Size | End Point | NSAID Dose | Results Relative Risk | Comments |
|---|---|---|---|---|---|---|
| Giovannucci *et al.* (1994) | Male health professionals (nonphysicians) 1986-1990 | 41,000<br>50 cases<br><br>21 cases | Colorectal cancer (incidence)<br>Colorectal cancer (metastatic disease or death) | Aspirin<br>≥ 2 times/week<br><br>≥ 2 times/week | 0.38<br>(0.18-0.78)<br>0.08<br>(0.01-0.59) | Adjusted for age, history of polyps, diet, smoking, alcohol, and physical activity |
| Giovannucci *et al.* (1995) | Nurses Health Study 1980-1992 | 47,900<br>501 cases | Colorectal cancer (incidence) | Aspirin<br>≥ 2 times/week<br>Duration of use<br>1-4 years<br>5-9 years<br>10-19 years<br>≥ 20 years | 0.62<br>(0.44-0.86)<br><br>1.06<br>(0.78-1.45)<br>0.84<br>(0.55-1.28)<br>0.70<br>(0.41-1.20)<br>0.56<br>(0.36-0.90) | Adjusted for age, history of polyps, diet, smoking, alcohol, and physical activity |

RR = relative risk (incidence rate among exposed/incidence rate among unexposed)

**Prevention of colon cancer in animal models with NSAIDs**

Beginning in 1980, numerous studies in rodents (15, 171, 207, 227-249) reported that treatment with several NSAIDs (indomethacin, sulindac, piroxicam, aspirin and others) effectively reduced tumor incidence and the growth of colonic polyps and cancer in response to a variety of chemical carcinogens (Table 2.6). Indomethacin has been the most actively investigated agent. In most cases, concomitant administration of the carcinogen and indomethacin resulted in a reduced number of animals with tumors, and a decreased number of tumors per animal compared with controls. Pollard and Luckert (228) showed that indomethacin caused a 40% reduction in 1,2-dimethylhydrazine-induced intestinal tumors in rats. Narisawa *et al.* (207) demonstrated that rats exposed to methylnitrosourea developed colonic tumors that could be significantly lowered by indomethacin. However, in rats given the carcinogen and then indomethacin, a rapid development of tumors occurred after 10-week cessation of treatment. Thus, the data suggested that indomethacin inhibited the development of colonic tumors but did not reject or kill the growing tumors. The influence of indomethacin and meclofenamate on the development of dimethylhydrazine-induced tumors in rats was examined by Metzger *et al.* (235). Colon cancer incidence was 56% in the indomethacin-treated animals, 90% in the meclofenamate-treated group and 88% in untreated control group. Piroxicam was shown to inhibit azoxymethane-induced colon cancer in rats in a dose-dependent manner (239). At a dose of 150 ppm piroxicam, the incidence of colon tumor was reduced by as much as 84% compared to untreated controls. In three studies (238, 243, 244), the combined therapy with D,L-α-difluoromethylornithine, an ornithine decarboxylase

Table 2.6. Effect of NSAIDs in animal models for colorectal cancer

| Authors | Year | NSAID | Animal | Carcinogen | Results |
|---|---|---|---|---|---|
| Kudo *et al.* | 1980 | Indomethacin | Rat | MAM | Dec. incidence and multiplicity |
| Pollard & Luckert | 1980 | Indomethacin | Rat | DMH | Dec. incidence, multiplicity and size |
| Narisawa *et al.* | 1981 | Indomethacin | Rat | MNU | Inhibition of development |
| Pollard & Luckert | 1981 | Indomethacin | Rat | DMH | Dec. multiplicity |
| Narisawa *et al.* | 1982 | Indomethacin | Rat | MNU | Inc. tumor number after drug stopped |
| Narisawa *et al.* | 1983 | Indomethacin | Rat | MNU | Inhibits promotion and initiation |
| Pollard & Luckert | 1983 | Indomethacin | Rat | DMH | Dec. tumor incidence |
| Pollard *et al.* | 1983 | Piroxicam | Rat | MAM | Dec. incidence and multiplicity |
| Sato *et al.* | 1983 | Indomethacin | Mouse | ------- | Inhibits adenomas growth in transplantable model |
| Metzger *et al.* | 1984 | Indomethacin/ Meclofenamate | Rat | DMH | Dec. incidence with indomethacin<br>No protective effect with meclofenamate |
| Narisawa *et al.* | 1984 | Indomethacin | Rat | AMMN | Dec. multiplicity |
| Narisawa *et al.* | 1984 | Indomethacin | Rat | MNU | $PGE_2$ failed to overcome protective effect of indomethacin |
| Pollard & Luckert | 1984 | Piroxicam | Rat | MNU | Dec. incidence and multiplicity |
| Nigro *et al.* | 1986 | Piroxicam | Rat | AOM | Dec. multiplicity, DFMO additive |
| Reddy *et al.* | 1987 | Piroxicam | Rat | AOM | Dose inhibitory response |
| Moorghen *et al.* | 1988 | Sulindac | Mouse | DMH | Dec. incidence and multiplicity |
| Ross *et al.* | 1988 | Piroxicam | Rat | ------- | Dec. tumor volume in transplantable model |

Table 2.6. (Continued)

| Authors | Year | NSAID | Animal | Carcinogen | Results |
|---|---|---|---|---|---|
| Pollard & Luckert | 1989 | Piroxicam | Rat | MAM | Dec. incidence and multiplicity |
| Moorghen *et al.* | 1990 | Sulindac | Mouse | DMH | Dec. microadenomas |
| Reddy *et al.* | 1990 | Piroxicam/ DFMO | Rat | AOM | Both agents additive dec. in cancer incidence, Single agents not effective versus adenomas |
| Rao *et al.* | 1991 | Piroxicam/ DFMO | Rat | AOM | Dec. incidence and multiplicity |
| Skinner *et al.* | 1991 | Sulindac | Rat | DMH | Dec. incidence and multiplicity; reversed with $PGE_1$ |
| Craven & DeRubertis | 1992 | Aspirin | Rat | DMH | Dec. incidence with aspirin given 1 week before and after carcinogen; No effect on incidence when started aspirin 4 weeks after carcinogen |
| Reddy *et al.* | 1993 | Aspirin | Rat | AOM | Dec. incidence and multiplicity |
| Davis *et al.* | 1994 | Aspirin | Rat | DMH | Dose inhibitory response |
| Alberts *et al.* | 1995 | Piroxicam Sulindac Sulindac sulfide | Rat | AOM | All agents inhibit tumor number |

AMMN, acetoxymethyl-methylnitrosamine; AOM, azoxymethane; DMH, dimethylhydrazine; MAM, methylazoxymethanol; MNU, methylnitrosourea; DFMO, D,L-α-difluoromethylornithine; Dec., decreased; Inc., increased.

inhibitor, and piroxicam showed an additive inhibitory effect on tumor formation. Sulindac was shown to have a protective effect against dimethylhydrazine-induced colonic tumors in the mouse. Sulindac administered with the carcinogen resulted in a significant reduction in tumor incidence and tumor burden, but sulindac administered 17 weeks after the carcinogen administration had no inhibitory effect (15). Craven and DeRubertis (246) examined the influence of aspirin on dimethylhydrazine-induced colonic carcinogenesis in rats. Incidence of adenocarcinomas was reduced 69% in rats receiving aspirin for 1 week before and after the carcinogen. However, aspirin had no effect on tumor incidence when started 4 weeks after carcinogen exposure, though the dosage suppressed *ex vivo* colonic $PGE_2$ production by 95%.

In summary, a variety of reports generated in chemical carcinogen tumor model support the concept that NSAIDs are effective in chemoprotection against rodent colorectal cancer. These agents appear to work at the initiation and promotion stages of carcinogenesis with different strengths of effect.

**NSAIDs and familial adenomatous polyposis (FAP)**

In 1983, Waddell and Loughry first reported that sulindac caused regression of rectal adenomatous polyps in 4 patients with familial adenomatous polyposis (13). FAP is an autosomal dominant inherited form of colorectal cancer in which patients develop hundreds to thousands of adenomas throughout the colorectum. Inevitably, colorectal cancer occurs, usually by the fifth decade of life. Waddell used sulindac (75-150 mg orally twice a day) in FAP patients and noted a striking 70-100% polyp regression in these persons. Following this initial account, similar observations of adenoma resolution with

oral sulindac in FAP patients have been reported by several investigators (Table 2.7) (12, 14, 206, 250-263). Polyp regression occurred in patients having various polyps and in those with intact colons and in persons with ileorectal anastomosis. In 1991, a randomized, placebo-controlled, double-blinded, crossover study in 9 FAP patients with ileorectal anastomosis was conducted by Labayle *et al.* (252). Patients received sulindac 300 mg/day, or placebo during two 4 months periods separated by a 1 month washout phase. With sulindac, a complete or almost complete regression of polyps was noted in all patients. With placebo, polyp numbers increased in 5 patients, remained unchanged in 2 patients and decreased in 2 patients. Differences in polyp numbers between sulindac and placebo groups were statistically significant. Nugent *et al.* reported a randomized controlled trial of sulindac in 24 FAP patients with subtotal colectomy and ileorectal anastomosis (255). Sulindac produced a statistically significant reduction in rectal polyp count; however, the reduction in duodenal polyp count was not statistically significant. In 1993, Giardiello *et al.* reported the results of a study of 22 patients with FAP including 18 patients without prior colectomy (253). Patients received oral sulindac 300 mg/day or placebo tablets for 9 months. A statistically significant decrease in mean polyp number and size occurred in patients treated with sulindac compared with placebo at 3, 6 and 9 months. When the patients were reexamined 3 months after cessation of the treatment, an increase in both the number and size of polyps was observed in sulindac-treated patients, although they did not return to the pretreatment baseline. A similar result was published by the same group in 1996 in which they further evaluate several clinical factors as possible predictors of sulindac effect on regression of colorectal adenomas on patients

Table 2.7. Effect of NSAIDs in human FAP patients

| Authors | Year | NSAID (Dose: mg/day) | Study design | Results |
|---|---|---|---|---|
| Waddell & Loughry | 1983 | Sulindac (150-300) | Case series | Regression of adenomas in 4 FAP patients |
| Gonzaga *et al.* | 1985 | Sulindac (Unspecified) | Case series | Regression of adenomas in 2 FAP patients |
| Waddell *et al.* | 1989 | Sulindac (300-400) | Case series | Regression of adenomas in 7 FAP patients |
| Friend | 1990 | Sulindac (150) | Case report | Regression of polyps in 3 Gardner's patients |
| Labayle *et al.* | 1991 | Sulindac (300) | Randomized-crossover | Regression of adenomas in 9 FAP patients |
| Rigau *et al.* | 1991 | Sulindac (400) | Case series | Regression of adenomas in 4 FAP patients |
| Giardiello *et al.* | 1993 | Sulindac (300) | Randomized | Regression of adenomas size and number<br>Both the number and size increased after sulindac was stopped (22 patients) |
| Hixson *et al.* | 1993 | Sulindac/ piroxicam (400)/(20) | Case series | Regression of adenomas in 3/5 patients on sulindac and 1 /2 adenomas patients on Piroxicam |
| Nugent *et al.* | 1993 | Sulindac (400) | Randomized | Regression of rectal but not duodenal adenomas in 24patients |
| Tonelli & Valanzano | 1993 | Sulindac (200) | Case series | Regression of adenomas in 8/13 FAP patients |

Table 2.7. (Continued)

| Authors | Year | NSAID (Dose: mg/day) | Study design | Results |
|---|---|---|---|---|
| Winde *et al.* | 1993 | Sulindac Suppository (300) | Case series | Regression of adenomas with rectal administration |
| Muller *et al.* | 1994 | Sulindac (300) | Case series | Regression of adenomas in 10 FAP patients, 1 juvenile polyp patients, 1 HNPCC patient |
| Niv & Fraser | 1994 | Sulindac (450) | Case report | Rectal cancer in FAP patient while on sulindac |
| Thorson *et al.* | 1994 | Sulindac (Unspecified) | Case report | Rectal cancer in FAP patient while on sulindac |
| Spagnesi *et al.* | 1994 | Sulindac (Unspecified) | Case series | Regression of adenomas in FAP patients |
| Debinski *et al.* | 1995 | Sulindac (400) | Randomized | Sulindac only caused regression of duodenal polyp with 2 mm or less in size |
| Giardiello *et al.* | 1996 | Sulindac (300) | Randomized | Regression of adenomas both in number and size in FAP patients |
| Hirota *et al.* | 1996 | Indomethacin Suppository (50-100) | Case report | Number of polyps decreased in 6/8 FAP patients; however, the number increased after indomethacin was discontinued |

FAP: familial adenomatous polyposis; HNPCC: hereditary nonpolyposis colorectal cancer

with FAP (263). They concluded sulindac treatment seems effective in producing regression of colorectal adenomas of FAP patients with previous subtotal colectomy regardless of baseline polyp number and size. Winde *et al.* and Hirota *et al.* (14, 256) applied rectal administration of sulindac or indomethacin to achieve polyp disappearance. Both of them suggested the rectal application may be useful in the future for adenoma regression because this procedure is uncomplicated, morbidity rates are low, and functional outcome is very good, even in the elderly. The effect of NSAIDs on sporadic colorectal adenoma regression (adenomas in patients without a hereditary form of colorectal cancer) has been evaluated in one small pilot study lasting 6 months. Hixson and co-workers used piroxicam (20 mg/day) in 2 individuals with adenomas up to 10 mm in size; only one polyp regressed partially (254). Of concern are several case reports of FAP patients maintained on sulindac who subsequently developed rectal cancer (259, 260).

In summary, studies evaluating FAP patients have clearly shown that NSAIDs cause the regression of adenomas polyps. This effect is fairly dramatic and occurs within months after therapy has been initiated. In addition to orally administered sulindac, rectal preparations also appear to be efficacious. The genetic defect and background in FAP patients clearly differs from patients with sporadic adenomas , and these genetic differences may have an impact on the effectiveness of NSAIDs in causing regression of adenomas which has not been well documented in patients with sporadic adenomas.

**Studies of colon cancer in *Min*/+ mouse models with NSAIDs**

Recently, a spontaneous intestinal tumor mouse model has been developed in

which C57BL/6J- *Min*/+ mouse, a strain containing a fully penetrant dominant mutation in the *Apc* tumor suppressor gene, is used to study FAP and NSAIDs treatment in experiments not possible with human patients (264-268). The mouse model allowed the investigators to precisely control environmental and genetic conditions in the development of neoplasia. All studies demonstrated that NSAIDs (piroxicam and sulindac) significantly reduced the tumor multiplicity of *Min*/+ mice (Table 2.8). In the sulindac study, three groups of mice were compared: normal C57BL/6J (+/+) mice with the wild-type *Apc* gene, the dominant mutant C57BL/6J- *Min*/+ mice, and the *Min*/+ mice treated with sulindac (at a dose of approximately 160 ppm in the drinking water beginning at 5-6 weeks of age) (265). Treatment with sulindac for approximate 9 weeks reduced average tumor number to 0.1 relative to the average of 11.9 tumors seen in untreated *Min*/+ mice. Since sulindac treatment was started at 5-6 weeks of age, the results presented in this study suggests sulindac affects tumor regression, rather than tumor initiation. To explain these results, these authors examined the intestinal expression of cyclooxygenase-2 (COX-2), as well as the levels of enterocyte apoptosis, in untreated mice (*Min*/+ and +/+) versus sulindac-treated *Min*/+ mice. Untreated *Min*/+ mice had increased expression of COX-2 protein, increased levels of $PGE_2$ in the intestine, and a 27-47% decrease in enterocyte apoptosis compared to +/+ animals. Sulindac treatment of *Min*/+ mice decreased COX-2 expression and $PGE_2$ production and restored apoptosis to levels similar to those observed in +/+ mice. In a study using piroxicam, the *Min*/+ mice were given the drug continually, via the diet, beginning at 30 days of age, and the tumor multiplicity was then assessed 6 weeks later (264). The results showed a dose-dependent decrease in intestinal adenomas

Table 2.8. Effect of NSAIDs in *Min*/+ mouse models

| Authors | *Min*/+ mice | NSAID | Results |
|---|---|---|---|
| Beazer-Barclay *et al.* (1996) | Male Female | Sulindac mixed with water 21 mg/l; 84 mg/l mixed with diet 167, 334 ppm | Sulindac (84 mg/l with water or 167 and 334 ppm with diet) significantly decreased tumor load |
| Boolbol *et al.* (1996) | Female | Sulindac mixed with water 0.5 ± 0.1 mg/day | Sulindac inhibited tumor formation and restored $PGE_2$ and apoptosis levels to baseline |
| Jacoby *et al.* (1996) | Female | Piroxicam mixed with diet 0, 50, 100, 200 ppm | Tumor multiplicity was decreased in a dose dependent response $TXB_2$ decreased in plasma with piroxicam treatment |
| Barnes & Lee (1998) | Male Female | Aspirin mixed with diet 250, 500 ppm | Aspirin decreased tumor multiplicity in small intestine but not the colon and reduced $PGE_2$ synthesis in control mice, whereas $PGE_2$ synthesis only was reduced significantly with 500 ppm but not 250 ppm aspirin in the *Min*/+ mice |
| Mahmoud *et al.* (1998) | Female | Sulindac sulfide Sulindac sulfone mixed with water 0.5 ± 0.1 mg/day | Sulfide significantly decreased tumor number and $PGE_2$ level in tissue Sulfone had a trend toward a decrease in tumor number but had no effect on $PGE_2$ level |

$PGE_2$: prostaglandin $E_2$; $TXB_2$: thromboxane $B_2$

and aberrant crypt foci as well as a parallel decrease in plasma level of thromboxane $B_2$ ($TXB_2$). The fact that tumor multiplicity was reduced even though treatment was not begun until 30 days of age suggest that piroxicam affects tumor promotion and /or maintenance rather than tumor initiation. In another study, tumor number was reduced by 40-70% in *Min*/+ mice given sulindac in either the drinking water or the diet beginning at approximate 30 days of age (266). Interestingly, a slightly stronger effect was observed when treatment was begun prenatally by treating pregnant females. This suggests that NSAIDs may be able to inhibit tumor formation and/or induce tumor regression. Alternatively, tumor growth may be more effectively inhibited by early administration of this drug. Mahmoud *et al.* reported the result of a study comparing the anti-tumor effect of sulindac sulfide, the active anti-inflammatory metabolite of sulindac, and sulindac sulfone, which has no anti-inflammatory activity (267). *Min*/+ mice were treated with either sulfide or sulfone (0.5 mg/day) in drinking water. *Min*/+ mice and homozygous C57BL/6J-+/+ normal litter-mates lacking the *Apc* mutation were used as controls. At 110 days of age, control *Min*/+ mice developed 33.2 ± 6.6 tumors per mouse compared to 0.6 ± 0.3 tumors for sulindac sulfide-treated *Min*/+ mice and 21.9 ± 4.5 tumors per mouse for sulfone-treated *Min*/+ mice. Decreased enterocyte apoptosis was observed in *Min*/+ control mice and *Min*/+ mice treated with sulfone. Sulfide restored apoptosis to normal level in the mucosa of *Min*/+ animals and decreased $PGE_2$ level in the small intestine of treated *Min*/+ animals by 59%. With respect to the efficacy of aspirin, wild type and *Min*/+ mouse were fed a diet supplementated with aspirin (250 or 500 ppm) or without aspirin for 7 weeks (268). Aspirin significantly reduced tumor multiplicity in the

small intestine, but not the colon, from an average of 35.8 tumors per mouse to 16 and 18.5 tumors per mouse with 250 and 500 ppm aspirin treatment, respectively. $PGE_2$ synthesis only was reduced significantly with 500 ppm aspirin in the *Min*/+ mice. In summary, the results of these studies lend further evidence that NSAIDs can have valuable chemopreventive and / or chemotherapeutic potential to protect from colorectal cancer.

## POSSIBLE MECHANISMS OF NSAIDs IN CANCER PREVENTION

The precise mechanisms explaining the chemopreventive and/or chemotherapeutic efficacy of NSAIDs against colorectal neoplasia are still unclear. Several hypotheses have been proposed.

### Hypothesis I: Cyclooxygenase as the target of NSAIDs

The commonly proposed hypothesis of the antitumor effect of NSAIDs is to inhibit cyclooxygenase activity and subsequently reduce the prostaglandin biosynthesis. Numerous studies have shown that prostaglandins are actively involved in cell growth and neoplastic cellular transformation. Many transformed cells produce more prostaglandins than normal cells and this excess amount of prostaglandins in turn can facilitate the growth of tumor cells. Enhanced prostaglandin production by naturally occurring or experimentally induced cancers has been documented in many studies (161, 269) (265). The mRNA of COX-2 was markedly elevated in colorectal adenocarcinomas and a subgroup of colonic adenomas as compared with levels in adjacent normal tissues, suggesting an increase in the expression of COX-2 may be involved in the progression of

tumor development (208). Enhanced COX-2 expression was also observed in patients clinically diagnosed with colorectal cancer (208-210). Prostaglandin synthesis inhibitors such as aspirin, indomethacin and sulindac have been demonstrated to inhibit cancer growth in laboratory animals, *in vitro* and in clinical studies (220, 247, 248, 270). Taken together, the role of COX-2 leading to increased $PGE_2$ production in colon cancers implies that the antitumor effect of NSAIDs on colon carcinogenesis may be mediated by inhibition of COX-2.

**Hypothesis II: Inhibition of cell growth by NSAIDs**

One proposed mechanism by which NSAIDs may affect tumorigenesis is their influence on the arachidonic acid cascade, whose products have been implicated in the initiation, promotion, growth and spread of tumors, as well as in the immune response to cancers. NSAIDs have been noted to affect cell proliferation in variety of experimental models, thus giving insights into their antitumor activity. For example, several NSAIDs have been shown to directly inhibit growth of rat hepatoma and human fibroblast cells in culture, and to suppress transplantable murine colon adenocarcinoma (234, 271). Indomethacin was also shown to arrest the G1-S progression in the cycle of cultured cells and reduce overall DNA synthesis (272, 273). The mechanism of this effect may be the inhibition of other cell cycle-related enzymes, for example, cAMP kinase and phosphodiesterases (274).

**Hypothesis III: Enhancement of immunosurveillance by NSAIDs**

NSAIDs have been shown to diminish growth of malignant cells by a direct effect on cells that enhances immune surveillance in the cancerous tissue. NSAIDs decrease

$PGE_2$ production and can stimulate cellular immune function both *in vitro* and *in vivo* (275). Treatments with NSAIDs has been shown to enhance a variety of immunological responses which may restore antitumor immunity in a compromised host. Prostaglandins play a role in host antitumor immunity by modulating a variety of immunological responses. For example, high levels of tissue $PGE_2$ are frequently associated with suppression of immune surveillance and killing of malignant cells. $PGE_2$ can act as a feedback inhibitor for cellular immune processes, such as T-cell proliferation, lymphokine production and cytotoxicity (276). In general, either increased production or increased sensitivity to normal amounts of $PGE_2$ results in depressed cellular immunity. Conversely, immune responses are generally enhanced by NSAIDs that inhibit prostaglandin synthesis, thus partially explaining the antitumor activity.

**Hypothesis IV: NSAID-induced apoptosis**

The proposal that NSAIDs may promote cell death is based on studies showing that prostaglandins are mitogenic, and that high concentrations of prostaglandins exist in certain tumors and tumor cell lines (161, 269). One hypothesis is that the anti-neoplastic activity of NSAIDs is mediated by their inhibition of prostaglandin synthesis. $PGE_2$ can induce apoptosis of both immature normal and malignant B-lymphocytes (277). This may suggest that $PGE_2$ secreting cells such as macrophage, which inhibit the B-cell microenvironments of lymphoid organs, may eliminate a subset of immature B-lymphocytes, and this may be important in controlling the spread of malignant lineages. There is the histologic evidence that treatment with mefenamic acid and diclofenac can

induce apoptosis in the crypts of colorectal biopsy tissue from patients who developed gastrointestinal irritation in response to NSAIDs (278, 279). Several animals and cell lines studies also demonstrated that sulindac can induce apoptosis in *Min*/+ mice and human colon HT-29 adenocarcinoma cell lines (265, 280, 281). Therefore, apoptosis has been suggested to be one consequence of NSAIDs' anti-neoplastic activity.

## RESEARCH OBJECTIVE

The commonly accepted hypothesis of the antitumor effect of NSAIDs is directly related to their ability to reduce eicosanoid biosynthesis by inhibiting cyclooxygenase activity. The overall objective of this dissertation is to assess the relationship of eicosanoids, particular prostaglandin $E_2$, to intestinal tumor load in the *Min*/+ mouse model.

To accomplish this objective, the following specific aims will be investigated:

Specific Aim 1: To characterize the tumor development (number and size) and eicosanoid ($PGE_2$, 6-keto $PGF_{1\alpha}$, and $LTB_4$) levels in the normal appearing intestinal tissue and tumors and to examine the potential effect of nonsteroidal anti-inflammatory drug (NSAID) sulindac on intestinal tumor load and tissue eicosanoid levels in the *Min*/+ mouse model.

Specific Aim 2: To investigate the temporal effect of NSAID sulindac on tumor load and the effects of sulindac on established tumors in the *Min*/+ mouse model.

Specific Aim 3: To evaluate the impact of enhancing eicosanoid biosynthesis using dietary arachidonic acid (AA) on tumor load with and without sulindac supplementation in the *Min*/+ mouse model.

Specific Aim 4: To study the effect of other NSAIDs, indomethacin and aspirin, on tumor load and correlate these effects to eicosanoid production generated from normal appearing intestinal tissue and tumors in the *Min*/+ mouse model.

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# PART III

## SULINDAC CAUSES RAPID REGRESSION OF PREEXISTING TUMORS IN *MIN*/+ MICE INDEPENDENT OF PROSTAGLANDIN BIOSYNTHESIS

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**ABSTRACT**

Several lines of evidence strongly link prostaglandins (PGs) and leukotrienes (LTs) to cancer of the intestine. Several studies have reported a 40-50% reduction in mortality from colorectal cancer in individuals who routinely consume nonsteroidal anti-inflammatory drugs, possibly by inhibiting cyclooxygenase activity. However, the role of eicosanoids in this process is still unclear. The heterozygote *Min*/+ mouse model, like patients with familial adenomatous polyposis, carries a nonsense mutation in the adenomatous polyposis coli (*APC*) gene that results in the spontaneous development of intestinal adenomas (100% incidence). This study investigated the association between eicosanoid biosynthesis, intestinal tumor load and the chemotherapeutic effect of the nonsteroidal anti-inflammatory drug sulindac during early and preexisting phases of tumor growth and development as well as residual effects after drug withdrawal. Administration of sulindac (320ppm) to *Min*/+ mice reduced the tumor number by 95%, but did not alter the levels of $PGE_2$ and $LTB_4$ in intestinal tissues. Increasing $PGE_2$ and $LTB_4$ levels by 44% with dietary arachidonic acid supplementation had no effect on tumor number or size. When sulindac was added to the arachidonic acid-supplemented diet, tumor number was reduced by 82%, whereas eicosanoid levels remained elevated. In *Min*/+ mice with established tumors, treatment with sulindac for 4 days reduced tumor number by 75% and continual administration of sulindac was necessary to maintain a reduced tumor load. In summary, alterations in eicosanoid formation were not correlated with tumor number or

size in the *Min*/+ mouse model; thus, the antitumor effect of sulindac seems to be PG independent.

## INTRODUCTION

Heterozygous C57BL/6J-Min (*Min*/+) mice are highly susceptible to spontaneous development of intestinal adenomas due to a germline mutation in one allele of the murine adenomatous polyposis coli (APC) gene with tumor initiation following loss of heterozygosity (1-3). The importance of this tumor suppressor gene to the phenotype has been recently confirmed by targeted mutagenesis (4, 5). The *Min*/+ mouse is a model for human intestinal tumorigenesis with similarities to an inherited form of human intestinal cancer, familial adenomatous polyposis (FAP). Individuals with FAP carry a mutation in one allele of the APC gene resulting in multiple intestinal adenomas following somatic mutation or loss of the second allele (2).

Several lines of evidence demonstrate an inverse relationship between the use of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and sulindac, and intestinal cancer. NSAIDs have been shown to reduce the relative risk of intestinal cancer in humans by 40-50% (6-14). Sulindac, in particular, decreases intestinal tumor load in *Min*/+ mice and FAP patients (12, 13) and inhibits chemically-induced colonic tumors in mice (15). Sulindac is a pro-drug and lacks anti-inflammatory activity until it is modified in the liver and by colonic bacteria to its metabolically active sulfide derivative (16). Since one of the major pharmacologic effects of NSAIDs is to inhibit cyclooxygenase (COX) activity, decreased prostaglandin (PG) biosynthesis is reasonably assumed to be an

important mechanism of action for these drugs. Boolbol *et al.* (17) have reported that the small intestine of *Min*/+ mice has elevated expression of COX-2 and increased production of $PGE_2$ compared to that of age-matched wild type controls. When treated with sulindac, they found that a reduction in tumor load was associated with a reduction in $PGE_2$ formation and COX-2 expression compared to untreated *Min*/+ mice. However, it has yet to be clearly established that the antitumor effect of sulindac is mediated via a reduction in prostaglandin biosynthesis. A number of studies suggest a prostaglandin-independent mechanism (18-21). For example, both sulindac metabolites (sulfide and sulfone derivatives) have similar anti-neoplastic effects; however, only the sulfide derivative inhibits cyclooxygenase (19).

Sulindac use is associated with regression of preexisting adenomas in FAP patients and its use is required for maintenance of a reduced tumor load in these individuals (22). Sulindac treatment in weanling *Min*/+mice results in dramatic reductions in tumor frequency. It appears that intestinal tumors in *Min*/+ mice are initiated early in life and that sulindac may be causing tumor regression in this model, perhaps by increasing apoptosis (17, 19, 23-24). However, studies to date have yet to establish that sulindac can cause regression of preexisting tumors in *Min*/+ mice. In a chemically-induced colonic tumor model, sulindac has a protective effect when provided concurrently with the carcinogen 1,2-dimethylhydrazine, but does not cause tumor regression (25). In the present study, we present data that shows that: (*a*) the antitumor effect of sulindac rapidly eliminates (within 4 days of treatment) macroscopic evidence of established tumors in

*Min*/+ mice; (*b*) this effect may be independent of prostaglandin modulation; and (*c*) withdrawal of the drug is associated with reappearance of tumors.

## MATERIALS AND METHODS

### Animals

Wild type (+/+) and *Min*/+ (*Apc*/+) male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), 35-40 days of age, were randomly divided into treatment groups (4-8 animals per group). They were housed in suspended stainless steel cages in a temperature controlled room (23±2°C) with a 12-h light-dark cycle and given free access to food and water. The health of the animals were checked daily. Prior to sacrifice, all animals were fasted over night. All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accord with the NIH *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

### Diets

The control (drug-free) diets were composed of purified AIN-93G powder diet (Dyets, Inc., Bethlehem, PA). Sulindac (Sigma Chemical Co., St. Louis, MO) was administered to the diets by thoroughly mixing 320 ppm sulindac with control diet. For those experiments using diets with modified fatty acid compositions, diets were supplemented with 1.5% (w/w) of the following fatty acid ethyl esters, oleic acid or arachidonic acid (Nu Chek Prep, Elysian, MN). All diets were prepared weekly and stored at -20°C. All animals were provided fresh food daily to prevent oxidation. Food

consumption was monitored daily and animal weights were recorded weekly and the actual drug doses were calculated according to food intakes.

**Experimental Design**

*Experiment 1:* Sixteen wild type (WT) male mice and sixteen male *Min*/+ mice were each divided into four groups of four animals upon arrival (approximately 35 days of age). One group each of wild type and *Min*/+ mice were sacrificed at 77, 101 and 115 days of age. The fourth group of wild type and *Min*/+ mice were treated with sulindac (S) (supplemented in the diet) at 320 ppm from the time of arrival through 115 days of age, at which time they were also sacrificed. Body weight and food consumption for all animals were monitored. At the time of sacrifice, tumor number, size and location were determined. Portions of normal appearing intestine and several of the largest tumors were harvested for histologic examination. The production of $PGE_2$, 6-keto-$PGF_{1\alpha}$ and $LTB_4$ from samples of normal appearing small intestine (mid-jejunum) were determined for each animal. Small intestinal tumors were also harvested from untreated 101 and 115 day old animals for $PGE_2$, 6-keto-$PGF_{1\alpha}$ and $LTB_4$ analysis.

*Experiment 2:* Twenty-six male *Min*/+ mice (35-40 days of age) were randomly divided into four groups upon arrival with (+S) or without (-S) sulindac supplementation. All animals were initially provided a sulindac-free diet until approximately 77 days of age (an average of 37 days). The animals in groups +S2 (n=7) and +S4 (n=8) were then provided sulindac (320 ppm) in the diet for 2 or 4 days prior to sacrifice, respectively. The animals in the +S20 group (n=5) were sacrificed following 20 days of sulindac supplementation. Tumor number, size and location were determined and samples were

harvested for histologic examination. A control group of *Min/+* mice not treated with sulindac was sacrificed at approximately 80 days of age (-S; n=6 ).

*Experiment 3:* Twelve male *Min/+* mice approximately 40 days of age were randomly divided into two groups upon arrival. One group (n=6) was provided a diet containing sulindac (320 ppm) from the time of arrival until 80 days of age, at which time they were switched to a sulindac-free diet for 40 days. At 120 days of age, the animals were sacrificed and tumor number, size and location were determined. The second group of *Min/+* mice (n=6) was also provided a diet containing sulindac from the time of their arrival (approximately 40 days of age) until 80 days of age and sacrificed at day 80 to provide tumor load data following 40 days of sulindac treatment.

*Experiment 4:* Twenty male *Min/+* mice approximately 35-40 days of age were randomly divided into four groups (5 animals/group) whose diets were supplemented (1.5% w/w) with the ethyl ester of oleic acid (18:1n-9, OA) or arachidonic acid (20:4n-6, AA), with or without sulindac (320 ppm). All mice were provided diet ad libitum until 60 days of age, at which time they were sacrificed and tumor number, size and location, tissue fatty acid composition, and eicosanoid production were determined.

**Gross and Histologic Examination**

At the scheduled age, the entire intestinal tract from each animal was removed and flushed with cold (4°C) phosphate buffered saline (PBS). Each intestinal tract was opened longitudinally and spread out, mucosal surface up, so that the location (large or small intes-tine), size and number of tumors could be determined using a dissecting microscope (18x). Tissue samples were fixed in 10% neutral buffered formalin (NBF). Five $\mu$m

paraffin embedded sections were stained with hematoxylin and eosin (H&E) for histological evaluation.

**Eicosanoids Measurement**

After sacrifice, normal-appearing intestinal sections (mid-jejunum) and pooled samples of individually excised small intestinal tumors were homogenized using a Polytron homogenizer in 0.1M cold Tris-HCl buffer (pH 7.4). *Ex vivo* production of eicosanoids was determined by incubating the homogenate for 15 min. The reaction was terminated by methanolic acidification following the addition of 8.8% of cold formic acid in methanol (final pH 3.5). Eicosanoids were isolated by solid phase extraction using an octadecyl ($C_{18}$) cartridge (Burdick & Jackson, Muskegon, MI) and eluted with 100% methanol. The recovery of eicosanoids was determined by adding 10 nCi [$^3$H]-$PGD_2$ to the sample as an internal standard prior to processing. Recoveries averaged 80% and were not statistically different among groups. The methanol was evaporated under an atmosphere of nitrogen and the extracts were resuspended in phosphate buffered saline (PBS), pH 7.4, containing 0.1% gelatin. $PGE_2$, 6-keto-$PGF_{1\alpha}$ and $LTB_4$ were measured by radioimmunoassay as described previously (26) using antiserum obtained from PerSeptive Diagnostics, Inc. (Cambridge, MA). All standards were purchased from Cayman Chemical (Ann Arbor, MI) and [$^3$H]-$PGE_2$, [$^3$H]-$PGD_2$, [$^3$H]-6keto-$PGF_{1\alpha}$ and [$^3$H]-$LTB_4$ were obtained from New England Nuclear (Boston, MA). An aliquot of the homogenate from each sample was used to determine protein concentration (27).

**Fatty Acid Analysis**

The fatty acid methyl esters of the tissue phospholipids were prepared and

analyzed as previously described (26). Briefly, tissues were homogenized in 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 thin layer chromatography on silica gel 60 HP-TLC plates (Merck, Darmatadt, 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 0.5 mM KOH in methanol for 8 min. at 86$^0$C. Following acidification with 0.7 mM HCl 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 $\mu$ film) (J&W Scientific, Folsom, CA). Separation was achieved by temperature programming from 160 to 250$^0$C at 3.5$^0$C/min with hydrogen as the carrier gas. The internal standard, pentadecanoic acid (15:0) methyl ester (100 $\mu$g) was added to each sample prior to the saponification process. The fatty acid methyl esters were identified by comparison of retention times with those of known standards.

**Statistical Analysis**

The Statistical Analysis System (SAS Institute, Inc, Cary, NC) was employed to analyze the data. Data were expressed as means ± SEM. Differences in body weight, food consumption, tumor number and biochemical parameters between the groups were analyzed statistically by a Students t-test or analysis of variance (ANOVA). Fisher's Least

Significant difference multiple comparison method was used to determine differences among groups. Data was considered significant at $p \leq 0.05$.

## RESULTS

### Food Intake and Body Weights

The general condition and health of *Min*/+ mice were not affected by sulindac treatment or dietary fatty acid manipulation. The *Min*/+ mice were approximately 10% smaller (by weight) than age-matched controls upon arrival (data not shown). Body weights of the wild type mice (WT), WT plus sulindac (WT+S) and *Min*/+ plus sulindac (Min+S) mice increased at similar rates throughout the study, but the rate of body weight gain in untreated *Min*/+ mice (Min) plateaued at approximately 77 days of age. The WT mice consumed significantly more food than the *Min*/+ mice independent of sulindac treatment (3.2±0.1g/d versus 2.8±0.1g/d). Although both *Min*/+ groups (Min and Min+S) consumed similar amounts of food, the rate of body weight gain by the sulindac-treated group paralleled that of the wild-type mice, while the untreated animals failed to gain weight after 77 days of age. We attribute this lack of weight gain in the untreated group to a greater tumor load in these animals as discussed below.

Food intake and body weight gain were not different between the oleic acid and arachidonic acid supplemented groups (data not shown).

### Effect of Age and Sulindac Treatment on Tumor Frequency, Size and Morphology

No tumors were found in the WT mice. At 77 days of age, *Min*/+ mice maintained on the control diet had approximately 40±5 tumors per animal with a mean tumor size of

1.2 mm (Table 3.1). The total tumor number remained relatively constant between 77 days and 115 days of age. Compared to the 77 day old mice, the average tumor size increased 43% and 52% at ages 101 days and 115 days, respectively. Overall, more than 95% of the tumors were located in the small intestines. There was no significant histologic distinction between the largest tumors harvested from 77, 101 or 115 day old Min/+ mice. In addition to features previously described (28), tumors frequently contained intraepithelial granulated leukocytes and scattered apoptotic bodies. Granulated cells had large brightly eosinophilic cytoplasmic granules and a round to indented hyperchromatic nucleus. These cells were not found in the normal intestinal mucosa outside of the tumors. There was frequently loss of at least a portion of the surface epithelium in larger tumors with exposure of the supporting stroma/lamina propria to the intestinal lumen. This tumor ulceration was far less common in the smaller lesions. Melena, frank intestinal hemorrhage, and splenomegally with marked erythropoiesis was most prominent in older animals carrying the heaviest tumor loads. This intestinal hemorrhage clearly results in a significant compensatory demand on body resources, as evidenced by the marked splenic erythropoiesis, and likely accounts in large part for the failure to gain weight as noted previously. When sulindac was supplemented to the diet for 80 days, tumor number and size were 93% and 55% lower, respectively, when compared to the unsupplemented 115 day old Min/+ mice (Table 3.1) and there was little to no evidence of melena or erythropoietic splenomegaly. Tumors from untreated and treated animals were histologically indistinguishable.

Table 3.1. Effect of age and sulindac on tumor size and number in the colon and small intestine of Min/+ mice

| | Age of *Min/+* mice (days)[a] | | | |
|---|---|---|---|---|
| | 77 (n=4) | 101 (n=4) | 115 (n=4) | 115+Sulindac (n=4) |
| | Total tumor number/group | | | |
| Colon | | | | |
| < 1 mm | 2[b] | 4 | - | -[c] |
| 1-1.5 | 2 | 6 | 1 | 1 |
| 2-2.5 | - | 1 | - | - |
| 3-3.5 | - | 2 | 1 | - |
| 4-4.5 | - | - | 1 | - |
| 5-5.5 | - | 2 | - | - |
| Small Intestine | | | | |
| < 1 mm | 61 | 9 | 4 | 8 |
| 1-1.5 | 74 | 86 | 82 | 3 |
| 2-2.5 | 14 | 65 | 63 | - |
| 3-3.5 | 5 | 6 | 9 | - |
| 4-4.5 | - | - | 3 | - |
| Total #/group | 158 | 181 | 164 | 12 |
| Ave. Tumor #/mouse[d] | 39.5±4.6[e] | 45.3±6.0[e] | 41.0±4.6[e] | 3.0±1.1[f] |
| Ave. Tumor Size[g] | 1.20±0.01[h] | 1.71±0.03[e] | 1.82±0.02[e] | 0.83±0.07[f] |

[a] *Min/+* mice were fed AIN-93G powder diet from arrival and sacrificed at 77, 101, and 115 days of age. One group was supplementated with 320 ppm sulindac from arrival through 115 days of age. Tumor number and size were examined from the entire intestinal tract using a dissecting microscope (18x).

[b] Number of tumors.

[c] None observed.

[d] Mean±SEM (n=4 animals/group).

[e] Values in each row with different superscript letters are significantly different by ANOVA ($p < 0.05$).

[f] Significantly different from age-matched (115 day old) non-sulindac treated *Min/+* mice by Student's *t* test ($p < 0.0001$).

[g] Average size (mm) was calculated as a weighted average for each size classification and expressed as Mean±SEM.

[h] Values in each row with different superscript letters are significantly different by ANOVA ($p < 0.05$).

**Temporal Effects of Sulindac on Established Tumors**

In order to determine if sulindac would cause regression of pre-existing tumors and, if so, how soon sulindac's effect would be observed, animals were provided a sulindac-free diet for an average of 37 days following arrival and then supplemented with sulindac for either 2, 4 or 20 days. When sulindac was administered for two days, the number of tumors identifiable under a dissecting microscope was 19% lower than in the age-matched *Min*/+control group which received no sulindac (29±3 vs 36±7 tumors) (Table 3.2). Within four days of sulindac treatment, tumor number (9±1) was 75% lower than the untreated group, with only a slight further reduction (7±2 tumors) after 20 days of sulindac treatment.

Under the dissecting microscope, small intestinal tumors from animals treated for 2 and 4 days were generally flatter than those in untreated animals and some had a slightly translucent, granular appearance. There were also scattered approximately 1-2 mm diameter crateriform lesions (an average of 10/mouse) in the small intestine. These depressions in the mucosal surface were lined by villi which were shrunken or absent centrally and had a white-frosted appearance around the perimeter of the lesion. Colonic tumors were not significantly different from those in untreated mice. After 20 days of sulindac treatment, tumors identified under the dissecting microscope were not significantly different from those of untreated mice.

Histologically, the tumors from animals treated for 2 days were flatter with a slight increase in villus architectural distinction, a reduction in the density of tubular-glandular structures, a relative increase in the amount of loose connective tissue between the glands,

Table 3.2. Temporal effects of sulindac on established tumors in the colon and small intestine of Min/+ mice

| | Treatment group[a] | | | |
|---|---|---|---|---|
| | -S (n=6) | +S2 (n=7) | +S4 (n=8) | +S20 (n=5) |
| | Total tumor number/group | | | |
| Colon | | | | |
| < 1 mm | -[b] | 1[c] | - | - |
| 1-1.5 | 4 | 4 | 1 | - |
| 2-2.5 | 3 | 3 | 2 | 2 |
| 3-3.5 | - | 1 | 1 | - |
| 4-4.5 | - | - | - | - |
| 5-5.5 | - | - | - | - |
| Small Intestine | | | | |
| < 1 mm | 58 | 74 | 34 | 15 |
| 1-1.5 | 132 | 119 | 35 | 17 |
| 2-2.5 | 20 | 4 | - | - |
| 3-3.5 | 1 | - | - | - |
| 4-4.5 | - | - | - | - |
| Total #/group | 218 | 206 | 73 | 34 |
| Ave. Tumor/mouse[d] | 36.3±7.3[e] | 29.4±2.7[e] | 9.1±0.8[f] | 6.8±1.6[f] |
| Ave. Tumor Size[g] | 1.14±0.01[e] | 0.99±0.01[f] | 0.92±0.03[f] | 0.89±0.02[f] |

[a] *Min*/+ mice were provided a sulindac-free diet for an average of 37 days following arrival. Groups of +S2, +S4, and +S20 were then provided with 320 ppm sulindac in the diet for 2, 4 or 20 days prior to sacrifice, respectively. Tumor number and size were examined from the entire intestinal tract using a dissecting microscope (18x).

[b] None observed.

[c] Number of tumors.

[d] Mean±SEM.

[e] Values in each row with different superscript letters are significantly different by ANOVA ($p < 0.05$).

[f] Values in each row with different superscript letters are significantly different by ANOVA ($p < 0.05$).

[g] Average size (mm) was calculated as a weighted average for each size classification and expressed as Mean±SEM.

and a more vertical orientation of the remaining glands. There was no evidence of significant increases in apoptosis or decreases in mitosis in H&E stained sections but there were occasional cystic glands filled with necrotic cellular debris. The crateriform lesions had no evidence of residual tumor cells and consisted of centrally shrunken, misshappen villi or ulceration surrounded by villi lined by epithelial cells with variably abundant cytoplasmic lipid droplets. Some of the tumors from animals treated for 4 days had similar changes to those seen after 2 days treatment with even more restoration of villus and crypt architecture. These lesions also contained intraepithelial granulated cells in some cases, similar to the untreated tumors. Other tumors in this 4 day treatment group showed less evidence of architectural normality or reduced tubular-glandular structures. Small cystic structures (single crypts) lined by proliferating epithelial cells were found incidentally in samples of small intestine from 2 and 4 day treated animals, resembling those occasionally seen in untreated Min/+ mice. Colonic tumors of treated animals were not significantly different from those of untreated animals.

**Residual Effect of Sulindac Treatment on Tumor Number and Size**

In order to determine if continued use of sulindac was required for maintenance of a reduced tumor load, animals were treated with sulindac for 40 days then placed on a sulindac-free diet for an additional 40 days. Following 40 days of sulindac supplementation (40-80 days of age), tumor number and size averaged 2.0±0.8 per mouse and 0.92±0.08 mm in diameter, respectively (Table 3.3). After a further 40 days on sulindac-free diet (80-120 days of age), the number of tumors increased to 28.8±6.0 per mouse with an average diameter of 1.14±0.03 mm.

Table 3.3. Residual effect of sulindac treatment on tumor Size and number in the colon and small intestine of Min/+ mice

| | Treatment group[a] | |
|---|---|---|
| | +S (n=6) | +S:-S(40) (n=6) |
| | Total tumor number/group | |
| Colon | | |
| < 1 mm | -[b] | - |
| 1-1.5 | 4[c] | - |
| 2-2.5 | - | 1 |
| 3-3.5 | - | 1 |
| 4-4.5 | - | 1 |
| 5-5.5 | - | 1 |
| Small Intestine | | |
| < 1 mm | 5 | 31 |
| 1-1.5 | 3 | 117 |
| 2-2.5 | - | 20 |
| 3-3.5 | - | 1 |
| 4-4.5 | - | - |
| Total #/group | 12 | 173 |
| Ave. Tumor/mouse[d] | 2.0±0.8[e] | 28.8±6.0[f] |
| Ave. Tumor Size[g] | 0.92±0.08[e] | 1.14±0.03[f] |

[a] *Min/+* mice were treated with 320 ppm sulindac in the diet for 40 days; one group (+S) was sacrificed to provide tumor load data after 40 days of sulindac treatment. The other group (+S:-S(40)) was then placed on a sulindac-free diet for an additional 40 days. Tumor number and size were examined from the entire intestinal tract using a dissecting microscope (18x).

[b] None observed.

[c] Number of tumors.

[d] Mean±SEM (n=6 animals/group).

[e] Values in each row with different superscript letters are significantly different by Student's *t* test ( $p < 0.05$).

[f] Values in each row with different superscript letters are significantly different by Student's *t* test ( $p < 0.05$).

[g] Average size (mm) was calculated as a weighted average for each size classification and expressed as Mean±SEM.

**Prostaglandin and Leukotriene Production by Normal Appearing Small Intestine and Tumors**

The production of $PGE_2$, 6-keto-$PGF_{1\alpha}$ and $LTB_4$ was not different in normal appearing intestines of wild type animals and the *Min/+* mice at 77, 101 or 115 days of age and was not reduced following sulindac treatment (Figs. 3.1-3.3). The small intestinal tumors of the *Min/+* mice produced significantly higher levels of $PGE_2$ and $LTB_4$ in the 101 and 115 day old mice compared to normal appearing small intestinal tissues (Figs. 3.1 and 3.2). 6-keto-$PGF_{1\alpha}$ production was not significantly different between the tumors and normal appearing intestines at 101 days of age, but was significantly lower at 115 days of age (Fig. 3.3).

**Effect of Dietary Arachidonic Acid on Intestinal Fatty Acid Composition, Eicosanoid Production, Tumor Frequency and Size**

Arachidonic acid was provided in the diet to enrich tissue phospholipids with arachidonic acid and augment eicosanoid biosynthesis and to evaluate what impact these changes would have on tumor frequency and/or size.

*Intestinal Fatty Acid Composition:* Tissue arachidonic acid levels in the arachidonic acid-supplemented groups were 60% higher compared to the oleic acid-supplemented groups, independent of sulindac treatment (Table 3.4). The increase in arachidonic acid levels occurred along with a concomitant decrease in linoleic acid levels, an effect typically observed with arachidonic acid feeding (26).

*Prostaglandin and Leukotriene Production:* Human and animal studies have clearly demonstrated that supplementing diets with arachidonic acid augments eicosanoid

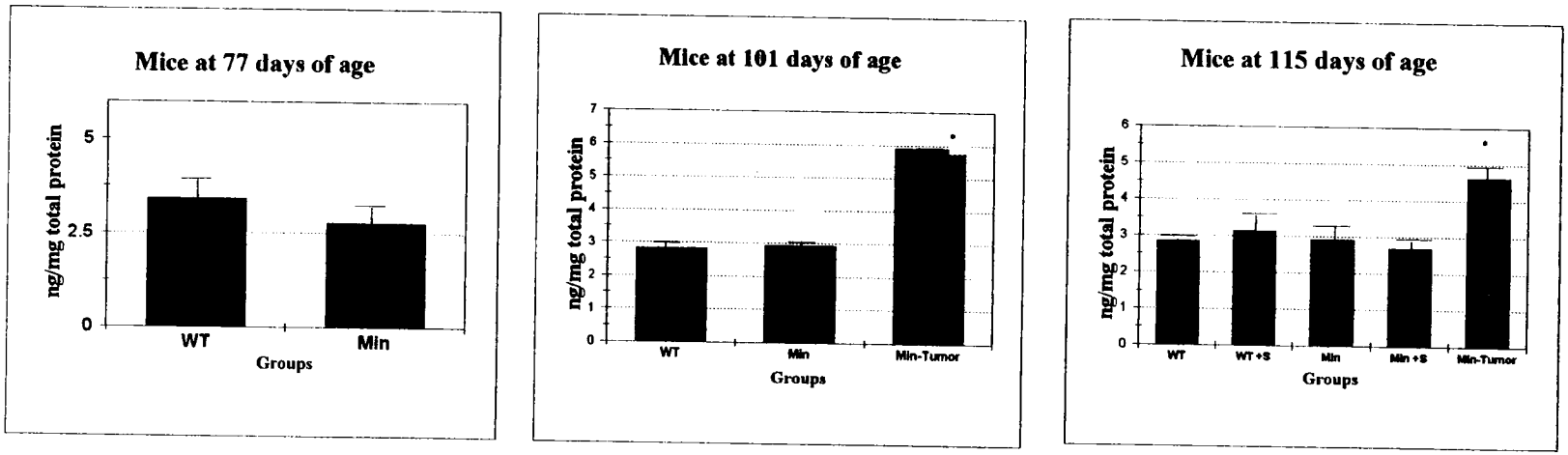


Fig. 3.1. $PGE_2$ production was not different in normal appearing intestinal sections from wild type (WT), *Min*/+ (Min), wild type plus sulindac (WT+S), *Min*/+ plus sulindac (Min+S) mice fed AIN-93G powder diet and sacrificed at 77, 101, and 115 days of age. However, small intestinal tumors from *Min*/+ (Min-Tumor) mice produced elevated amounts of $PGE_2$. Columns represent the means of four experimental values; bars, SEM. * significantly different from all other groups, $p < 0.05$.

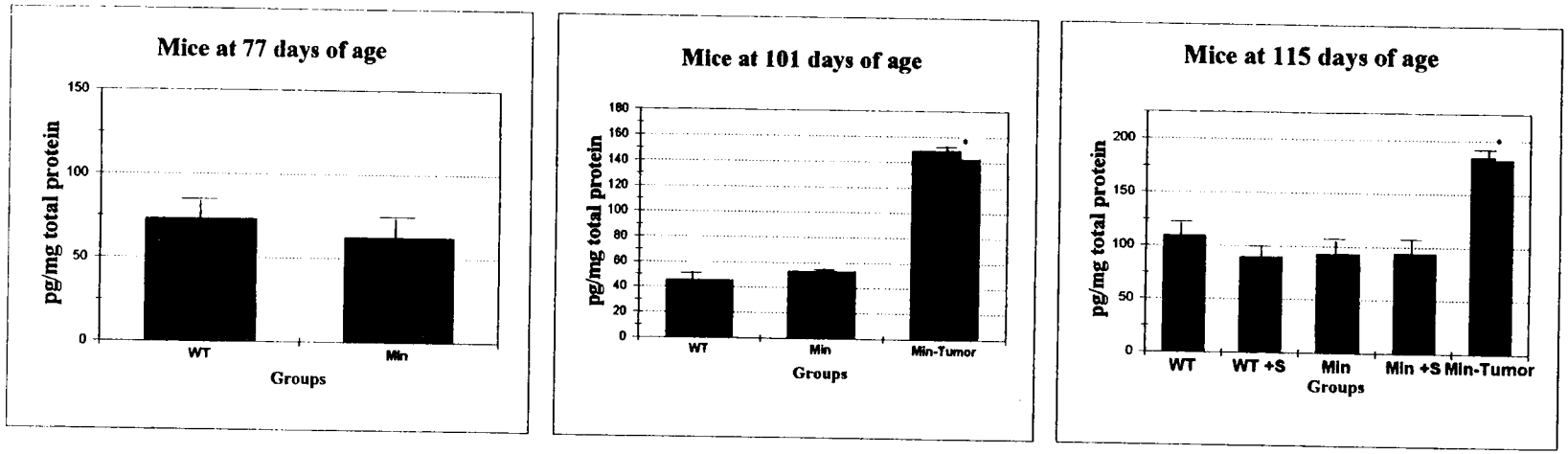


Fig. 3.2. $LTB_4$ production was not different in normal appearing intestinal sections from wild type (WT), *Min*/+ (Min), wild type plus sulindac (WT+S), *Min*/+ plus sulindac (Min+S) mice fed AIN-93G powder diet and sacrificed at 77, 101, and 115 days of age. However, small intestinal tumors from *Min*/+ (Min-Tumor) mice produced elevated amounts of $LTB_4$. Columns represent the means of four experimental values; bars, SEM. * significantly different from all other groups, $p < 0.05$.

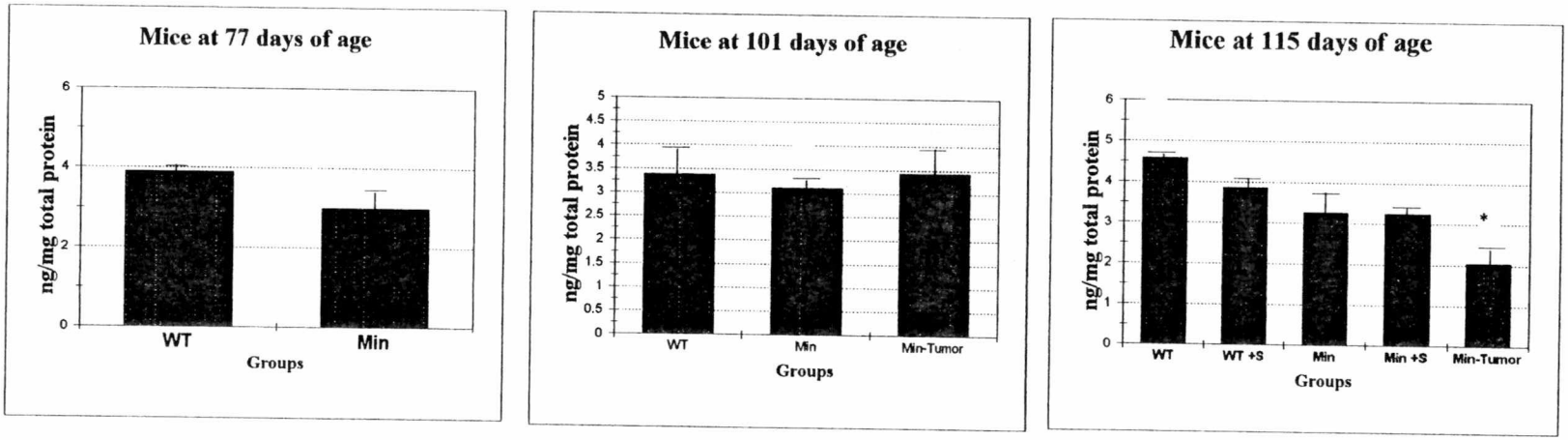


Fig. 3.3. 6-keto-$PGF_{1\alpha}$ production was not different in normal appearing intestinal sections from wild type (WT), *Min*/+ (Min), wild type plus sulindac (WT+S), *Min*/+ plus sulindac (Min+S) mice fed AIN-93G powder diet and sacrificed at 77, 101, and 115 days of age. Small intestinal tumors from *Min*/+ (Min-Tumor) mice did not produce significantly different amounts of 6-keto-$PGF_{1\alpha}$ at 101 days of age but the amount was significantly lower at 115 days of age compared to normal appearing intestines. Columns represent the means of four experimental values; bars, SEM. * significantly different from other groups, $p < 0.05$.

Table 3.4. Small intestine phospholipid fatty acid composition of Min/+ mice whose diets were supplemented with dietary arachidonic acid or oleic acid and with or without sulindac

| Selected fatty acids[b] | Treatment group[a] | | | |
|---|---|---|---|---|
| | OA (n=5) | OA+S (n=5) | AA (n=5) | AA+S (n=5) |
| 18:2 (n-6) | 23.11±1.09[c] | 20.73±0.93[c] | 15.21±0.79[d] | 16.03±0.49[d] |
| 20:4 (n-6) | 15.71±0.29[d] | 15.73±0.32[d] | 24.62±0.78[c] | 25.66±0.36[c] |

[a] *Min/+* 35-40 day old mice were fed AIN-93G powder diet supplemented with 1.5% (w/w) ethyl esters of oleic acid or arachidonic acid with 320 ppm sulindac (OA+S; AA+S) or without sulindac (OA; AA) until 60 days of age. Phospholipids were separated by TLC and methylated using ethereal diazomethane and the fatty acid methyl esters were then analyzed via gas chromatography.

[b] Data are mol% and summarized as Mean±SEM (n=5 animals/group).

[c] Values in each row with different superscript letters are significantly different by ANOVA ($p < 0.05$).

[d] Values in each row with different superscript letters are significantly different by ANOVA ($p < 0.05$).

biosynthesis systemically, and as such, provides an effective non-invasive technique to elevate prostaglandins and leukotrienes in vivo (26, 29-31). The feeding of arachidonic acid resulted in a significant increase (approximately 42% overall) in the production of $PGE_2$, 6-keto-$PGF_{1\alpha}$ and $LTB_4$. Treatment with sulindac had no effect on reducing eicosanoid production, in particular, the prostaglandins $PGE_2$ and 6-keto-$PGF1_{\alpha}$ (Fig. 3.4).

*Tumor Frequency and Size:* Arachidonic acid supplementation had no effect on tumor number compared to oleic acid, but the average size of the tumors were 15% smaller (Table 3.5). Sulindac treatment reduced the number of tumors by 96% and 83% in the oleic acid and arachidonic acid groups, respectively. The average size of the tumors in the sulindac-treated animals were not different among the arachidonic acid and oleic acid-supplemented groups.

## DISCUSSION

The objectives of this study were to evaluate the progression of the *Min*/+ phenotype over time, evaluate the temporal effect of the NSAID sulindac as a chemopreventive or therapeutic agent for intestinal tumors, and determine whether alterations of eicosanoid biosynthesis are related to sulindac's effect.

*Min*/+ mice averaged 42 intestinal tumors/mouse over the course of the study (Table 1), which is consistent with other laboratories where genetically similar *Min*/+ mice (i.e., on a C57BL/6J background) develop an average of 30-60 macroscopically visible tumors by the age of 90 days (32-34). Our results suggest, under normal conditions

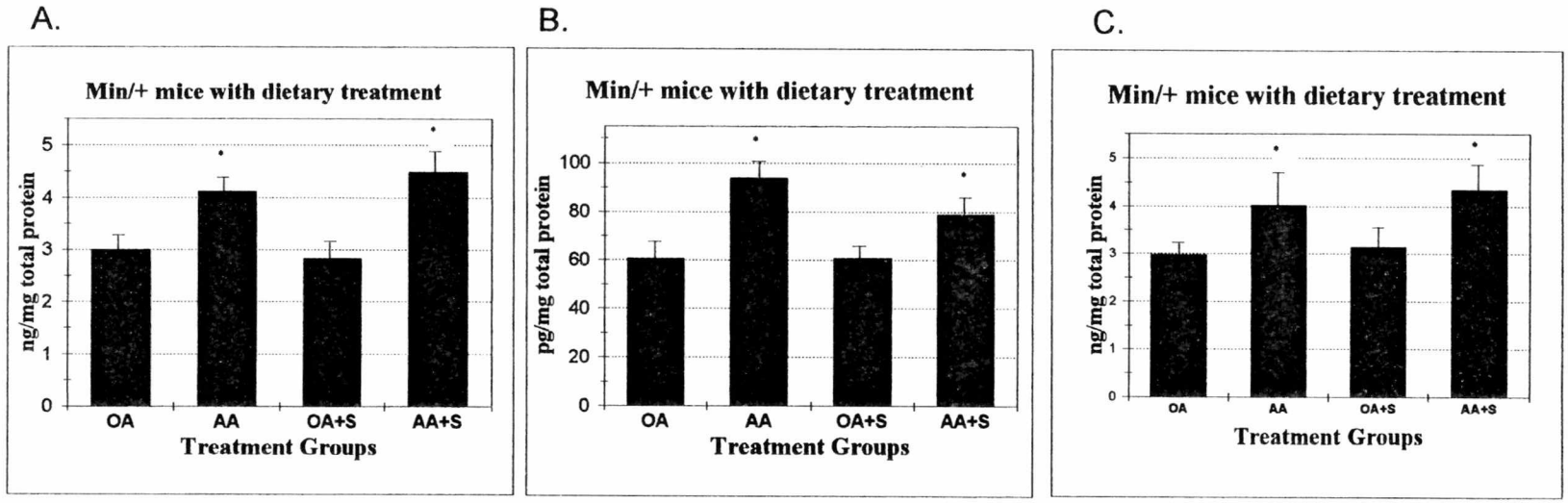


Fig. 3.4. Eicosanoid production of normal appearing intestinal section from *Min/+* mice supplemented with 1.5% (w/w) ethyl esters of oleic acid or arachidonic acid with sulindac (OA+S; AA+S) or without sulindac (OA; AA). Supplementation of AA resulted in significantly higher amounts of $PGE_2$ (A), $LTB_4$ (B), and 6-keto-$PGF_{1\alpha}$ (C) compared to the respective OA controls. Sulindac treatment failed to reduce eicosanoid formation. Columns represent the means of five experimental values; bars, SEM. * significantly different from respective control group at $p < 0.05$ (AA *vs.* OA; AA+S *vs.* OA+S).

Table 3.5. Tumor size and number in the colon and small intestine of Min/+ mice supplemented with arachidonic acid or oleic acid and with or without sulindac treatment

| | Treatment group[a] | | | |
|---|---|---|---|---|
| | OA (n=5) | OA+S (n=5) | AA (n=5) | AA+S (n=5) |
| | Total tumor number/group | | | |
| Colon | | | | |
| < 1 mm | -[b] | - | - | - |
| 1-1.5 | 8[c] | 4 | 1 | 3 |
| 2-2.5 | - | - | - | - |
| 3-3.5 | 1 | - | - | - |
| 4-4.5 | - | - | - | - |
| 5-5.5 | - | - | - | - |
| Small Intestine | | | | |
| < 1 mm | 84 | 4 | 102 | 18 |
| 1-1.5 | 103 | 1 | 66 | 8 |
| 2-2.5 | 5 | - | 2 | - |
| 3-3.5 | - | - | - | - |
| 4-4.5 | - | - | - | - |
| Total #/group | 201 | 9 | 171 | 29 |
| Ave. Tumor/mouse[d] | 40.2±3.1[e] | 1.8±0.6[f] | 34.2±6.4[e] | 5.8±0.4[f] |
| Ave. Tumor Size[g] | 0.97±0.01[e] | 0.9±0.1[ef] | 0.82±0.01[f] | 0.79±0.05[f] |

[a] *Min*/+ 35-40 day old mice were fed AIN-93G powder diet supplemented with 1.5% (w/w) ethyl esters of oleic acid or arachidonic acid with 320 ppm sulindac (OA+S; AA+S) or without sulindac treatment (OA; AA) until 60 days of age. Tumor number and size were examined from the entire intestinal tract using a dissecting microscope (18x).

[b] None observed.

[c] Number of tumors.

[d] Mean±SEM (n=5 animals/group).

[e] Values in each row with different superscript letters are significantly different by ANOVA ($p < 0.05$).

[f] Values in each row with different superscript letters are significantly different by ANOVA ($p < 0.05$).

[g] Average size (mm) was calculated as a weighted average for each size classification and was expressed as Mean±SEM.

intestinal tumor initiation has occurred by 60 days of age based on two features: (1) the average number of tumors per mouse did not increase between 60 and 115 days of age (see Tables 1 and 5) and; (2) the relative number of tumors less than 1mm in size are reduced as the animals aged with an equivalent increase in those greater than 1mm in diameter. This shift in tumor size is consistent with growth of pre-existing tumors and minimal induction of new tumors as the animal ages. Tumor initiation before 60 days of age is in agreement with data generated by Shoemaker *et al.* (33).

Consistent with the results of other studies (17, 34), treatment of *Min*/+ mice with sulindac shortly after genetic screening resulted in stark reductions in tumor frequency. With withdrawal of sulindac treatment, tumor frequency increased indicating an obligate requirement of sulindac treatment for this anti-tumor effect. We also demonstrated similar results in *Min*/+ mice with pre-existing macroscopically evident tumors. We demonstrated a maximum impact of sulindac's effect on pre-existing tumors within four days of treatment. Tumors, 2 and 4 days post-treatment, differed slightly, both macroscopically and histologically, when compared to tumors of untreated animals. The tumors from treated animals appeared flatter with some loss of tubular and glandular structures and more obvious vertically oriented crypts suggesting reappearance of a more normal small intestinal architecture. Apoptosis was not clearly increased in H&E stained tumors from animals treated with sulindac for 2 days. This does not, however, rule out the possibility of a role for apoptosis in regression of these lesions as it was apparent that considerable changes had already taken place by the time these tissues were harvested. It is entirely possible that elimination of apoptotic neoplastic cells had already occurred

through phagocytosis or desquamation into the intestinal lumen following 2 days of treatment. In addition, evaluation of H&E stained sections may not be the most sensitive technique for detection of cells undergoing this process.

While the mechanisms by which sulindac causes regression of adenomas has yet to be clarified, the drug has been shown to induce apoptosis, possibly by inhibiting COX-2 (17-18, 23). Over-expression of COX-2 has been observed in established *Min*/+ mouse tumors (35) and has been linked to inhibition of apoptosis (17-18, 23, 35, 36). Therefore, inhibition of prostaglandin biosynthesis is a convenient explanation for sulindac's effects. However, a growing body of contradictory evidence suggests the possibility of a prostaglandin-independent mechanism.

Prostaglandin and leukotriene production was not different in normal appearing intestinal tissue from *Min*/+ mice compared to their wild type litter mates at any age. We did, however, find that tumors produced significantly higher levels of $PGE_2$ and $LTB_4$ at 101 and 115 days of age and significantly lower levels of 6-keto-$PGF_{1\alpha}$ at 115 days of age compared to normal appearing intestine, but it is still not clear as to whether these are important in tumor growth. There appears to be an antithetic relationship between the levels of $PGE_2$ and $PGI_2$ (measured as 6-keto-$PGF_{1\alpha}$) in tumor cell lines and neoplastic colonic tumors in humans (37, 38). Increasing eicosanoid biosynthesis as a result of dietary manipulation with arachidonic acid had no effect on tumor frequency and did not result in larger tumors. However, it is possible that basal eicosanoid production exceeds the minimum threshold for effecting tumor initiation/promotion and any effects on tumor load are observed only after inhibiting eicosanoid biosynthesis. In addition, our eicosanoid

data does not fully address the involvement of COX-2. Tumors over-express COX-2 in humans and *Min*/+ mice compared to adjacent normal-appearing tissue and this may account, in part, for the increased production of $PGE_2$ (35, 39, 40). Histologic examination demonstrated that tumors were infiltrated by intra-epithelial granulated leukocytes which may be a source of the elevated levels of $PGE_2$ and $LTB_4$ observed in this study. While it has been reported that normal appearing intestinal tissue from *Min*/+ mice produces significantly higher levels of $PGE_2$ and over-express COX-2 compared to wild type litter mates (17), others have more recently reported that COX-2 is not expressed in normal appearing intestine in both *Min*/+ and the *APC*-716 mouse model (a transgenic strain of Min mice) (35, 36). These recent data are more consistent with our results and, as such, eicosanoid levels in normal small intestines of *Min*/+ and wild type mice can not be used to distinguish the *Min*/+ phenotype.

In addition to the sulfide metabolite of sulindac which has anti-COX activity, the drug is also oxidized *in vivo* to its sulfone derivative (41). Sulindac sulfone has been shown to be a relatively ineffective inhibitor of either COX-1 or -2 (16), but has antiproliferative effects and induces apoptosis in various tumor cell lines (19, 21). Recently, the sulfone derivative was also shown to be effective in inhibiting chemically-induced colonic and mammary carcinogenesis (20, 28) and colonic preneoplastic lesions (42) without inhibiting prostaglandin biosynthesis, raising the question as to whether sulindac is acting via inhibition of prostaglandins. It is currently unclear whether sulindac sulfone is contributing to the antitumor effect of sulindac in *Min*/+ mice.

In summary, sulindac acts very quickly (within 4 days) to cause regression of small

intestinal tumors in *Min/+* mice, while long term effectiveness requires constant use of the drug. Although the mechanism by which sulindac decreases tumor load is unclear, its ability to inhibit COX-2 activity and/or decrease COX-2 expression is believed to be related to induction of apoptosis which likely has a role in the regression process. Our data suggests that tumor load is not correlated with eicosanoid production and that the ability of sulindac to reduce tumor load in the Min/+ mouse model does not appear to be dependent upon the inhibition of prostaglandins, in particular, $PGE_2$.

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# PART IV

## DISCORDANT EFFECT OF ASPIRIN AND INDOMETHACIN ON INTESTINAL TUMOR LOAD IN *MIN/+* MICE

**This manuscript has been submitted to publish as Advances in Brief with co-authors McEntee, M.F., Whelan, J. in Cancer Research**

## ABSTRACT

Epidemiologic and animal studies indicate that sustained use of NSAIDs have a chemopreventive effect against the incidence of colorectal neoplasia and subsequent mortality. We previously demonstrated that sulindac significantly reduces intestinal tumor load in *Min/+* mice apparently independent of prostaglandin biosynthesis. In the present study, we show that indomethacin (9 ppm) is a very potent chemopreventive agent reducing tumor load by 85% and significantly inhibiting basal and *ex vivo* prostaglandin formation ($p < 0.006$ and $p < 0.0001$, respectively). Aspirin (400 ppm) has a similar impact on prostaglandin levels, but in contrast to indomethacin, is uneffective in reducing the tumor load. The data indicate a discordance between the impact of different NSAIDs and their anti-neoplastic activity.

## INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) reduce chemically-induced and genetically predetermined colorectal tumors in experimental animal models and the risk of developing colorectal cancer in humans (1-8). Aspirin is the most widely used NSAID and has a unique mechanism of action in inhibiting cyclooxygenase activity (9). Whereas most epidemiological studies report a 40-50% reduction in the incidence of colorectal cancer with regular aspirin use, some studies report that the incidence of colorectal cancer actually increases slightly in aspirin users (10, 11). However, prospective clinical trials designed to specifically investigate the effectiveness of aspirin as a therapeutic agent on intestinal tumors have not been done in spite of the epidemiologic evidence supporting its

efficacy (12). In clinical trials, the NSAID sulindac is very effective in reducing tumor number and size in famalial adenomatous polyposis (FAP) patients, individuals who carry a germline mutation in the tumor suppressor gene adenomatous polyposis coli (*APC*) (13, 14). Similarly, sulindac reduces intestinal tumor load by 90-95% and the number of pre-existing tumors by 80% in *Min/+* mice, an animal model for FAP that also carries a germline mutation in murine *APC* (6). While NSAIDs like sulindac and piroxicam (5) are very effective in reducing tumor number in *Min/+* mice, there is recent evidence suggesting aspirin may not be as effective as other NSAIDs in this model (15). This study was designed to evaluate the efficacy of aspirin and indomethacin as chemopreventive agents on intestinal tumors in *Min/+* mice.

## MATERIALS AND METHODS

### Animals

Male C57BL/6J *Min/+* (*Apc/+*) mice were obtained at 35 days of age (Jackson Laboratories, Bar Harbor, ME) and were randomly assigned into treatment groups (4-5 animals per group) at the time of arrival. Mice had free access to food and water at all times and their health was checked daily. All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accord with the NIH *Guide for the Care and Use of Laboratory Animals* (NRC 1985).

### Diet

The control (drug-free) diets were composed of purified AIN-93G powder diet (Dyets, Inc., Bethlehem, PA). Experimental diets were prepared weekly by thoroughly

mixing 400 ppm aspirin or 9 ppm indomethacin (Sigma Chemical Co., St. Louis, MO) with the control diet. Diets were stored at -20°C and all animals were provided fresh food daily. Food consumption was monitored daily and animal weights were recorded weekly. Drug doses were calculated based on actual food consumed.

**Experimental Design:**

*Experiment 1:* Twelve male C57BL/6J *Min/+* (*Apc/+*) mice were assigned to three groups (drug-free, aspirin (ASA) or indomethacin), four animals per group. All mice were maintained on respective diets to 80 days of age, at which point, tumor number, size and location were determined as previously described (6). The production of $PGE_2$ and 6-keto-$PGF_{1\alpha}$ from samples of normal appearing small intestine were determined for each animal.

*Experiment 2:* Ten male C57BL/6J *Min/+* (*Apc/+*) mice were randomly assigned to two groups (drug-free or aspirin), five animals per group and treated as described above to confirm the aspirin results from experiment 1.

**Eicosanoids Measurement**

Intestinal mucosa was homogenized using a Polytron homogenizer in 0.1M cold Tris-Hcl buffer (pH 7.4). An aliquot of the homogenate from each sample was used to determine protein concentration with a modified Lowry assay. The production of eicosanoids was determined in intestinal mucosa homogenates by incubating them at 37°C for 0 (basal level) and 15 min (*ex vivo* production). The incubation was stopped by adding indomethacin (final conc. 1mM), followed by adding 8.8% of cold formic acid in methanol (final pH 3.5). Eicosanoids were isolated by solid phase extraction using an octadecyl

($C_{18}$) cartridge (Burdick & Jackson) and eluted with 100% methanol. The recovery of eicosanoids was determined by adding 10 nCi [$^3$H]-$PGD_2$ to the sample as an internal standard prior to processing. The methanol was evaporated under an atmosphere of nitrogen and the extracts were resuspended in phosphate buffered saline (PBS), pH 7.4, containing 0.1% gelatin. $PGE_2$ and 6-keto-$PGF_{1\alpha}$ were measured by radioimmunoassay as described previously (6) and according to the manufacture's instruction with antiserum obtained from PerSeptive Diagnostics, Inc. (Cambridge, MA). 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).

**Statistical Analysis**

The Statistical Analysis System (SAS Institute, Inc, Cary, NC) was employed to analyze the data. Data were expressed as means ± SEM. Differences in tumor load (number and size) and biochemical parameters between the groups were analyzed statistically by analysis of variance (ANOVA). The Fisher least significant difference at $p \leq 0.05$ was tested to compare means.

## RESULTS

**Dietary Intake and Body Weights**

Growth rates corresponded to food intake. Food intake and growth rates among the *Min*/+ mice groups were similar, independent of drug treatment (data not shown). There was no evidence of toxicity due to aspirin or indomethacin treatment in any of the mice.

**Effect of Aspirin and Indomethacin on Tumor Number and Size**

The untreated *Min*/+ mice developed an average of 29±5.6 tumors by 80 days of age, whereas those supplemented with aspirin or indomethacin developed 50±13.6 and 4.5±0.7 tumors/mouse, respectively (one animal on aspirin treatment developed 89 intestinal tumors) (Table 1). Tumor number was not significantly different among the control and aspirin-treated groups. The average size of the tumors was unaffected by any treatment (Table 1). A second experiment confirmed that aspirin treatment had no effect on reducing the tumor number and size compared to untreated *Min*/+ mice (*i.e.* 32.6±9.5 vs. 32.0±5.7) (Table 2).

**Prostaglandin Formation**

Basal and *ex vivo* eicosanoid production were evaluated in normal appearing intestines from drug-free *Min*/+ mice and *Min*/+ mice treated with aspirin or indomethacin.

Basal levels of $PGE_2$ and 6-keto-$PGF_{1\alpha}$ were significantly lower in the animals treated with indomethacin compared to untreated controls (Table 1). *Ex vivo* $PGE_2$ and 6-keto-$PGF_{1\alpha}$ production was also significantly lower in both the aspirin and indomethacin treated groups compared to untreated controls (Table 1). In the second aspirin study, basal levels and *ex vivo* production of $PGE_2$ and 6-keto-$PGF_{1\alpha}$ were again significantly lower in the aspirin-treated mice compared to untreated *Min*/+ mice (Table 2).

## DISCUSSION

NSAIDs have been shown to be effective chemopreventive agents for a number of

Table 4.1. Tumor load and eicosanoid production from normal appearing intestine in *Min/+* mice treated with aspirin or indomethacin

| | Treatment group[1] | | |
|---|---|---|---|
| | Untreated (n=4) | Indomethacin (n=4) | Aspirin (n=4) |
| Tumors | | | |
| Avg. Tumor #/mouse[2] | 29.3±5.6[a] | 4.5±0.7[b] | 50±13.6[a] |
| Avg. Tumor Size[3] | 1.18±0.05[a] | 1.16±0.11[a] | 1.10±0.03[a] |
| Eicosanoids[4] | | | |
| Basal level (ng/mg total protein) | | | |
| $PGE_2$ | 0.74±0.12[a] | 0.46±0.04[b] | ND[5] |
| 6-keto-$PGF_{1\alpha}$ | 0.64±0.11[a] | 0.18±0.02[b] | ND |
| *ex vivo* production (ng/mg total protein) | | | |
| $PGE_2$ | 6.94±1.65[a] | 1.87±0.66[b] | 1.11±0.07[b] |
| 6-keto-$PGF_{1\alpha}$ | 4.65±0.92[a] | 0.16±0.26[b] | 1.39±0.10[b] |

[1] *Min/+* mice were fed purified AIN-93G powder diet from arrival through 80 days of age. NSAIDs were administered by thoroughly mixing 400 ppm aspirin or 9 ppm indomethacin with control diet. Tumor number and size were examined from the entire intestinal tract using a dissecting microscope (18x).

[2] Mean±SEM.

[3] Average size (mm) was calculated as a weighted average for each size classification and expressed as mean±SEM.

[4] ng/mg total protein

[5] Not determined (see experiment 2)

[a,b] Values in each row with different superscript letters are significantly different by ANOVA, at $p < 0.05$.

Table 4.2. Tumor load and eicosanoid production from normal appearing intestine in *Min*/+ mice treated with or without aspirin

| | Treatment group[1] | |
|---|---|---|
| | Untreated (n=5) | Aspirin (n=5) |
| Tumors | | |
| Avg. Tumor #/mouse[2] | 32.6±9.5[a] | 32.0±5.7[a] |
| Avg. Tumor Size[3] | 1.04±0.09[a] | 1.10±0.04[a] |
| Eicosanoids[4] | | |
| Basal level (ng/mg total protein) | | |
| $PGE_2$ | 1.62±0.27[a] | 0.40±0.08[b] |
| 6-keto-$PGF_{1\alpha}$ | 0.62±0.12[a] | 0.15±0.02[b] |
| *ex vivo* production (ng/mg total protein) | | |
| $PGE_2$ | 6.91±0.36[a] | 2.02±0.16[b] |
| 6-keto-$PGF_{1\alpha}$ | 3.57±0.27[a] | 1.07±0.08[b] |

[1] *Min*/+ mice were fed purified AIN-93G powder diet from arrival through 80 days of age. Aspirin was administered by thoroughly mixing 400 ppm aspirin with control diet. Tumor number and size were examined from the entire intestinal tract using a dissecting microscope (18x).

[2] Mean±SEM.

[3] Average size (mm) was calculated as a weighted average for each size classification and expressed as mean±SEM.

[4] ng/mg total protein

[a,b] Values in each row with different superscript letters are significantly different by ANOVA, at $p < 0.05$.

cancers, including colorectal cancer, and it has been presumed that this is due to their ability to inhibit prostaglandin formation (2, 9, 16). Aspirin use, in particular, reduces the relative risk of intestinal cancer in humans by 40-50% (7, 8) and inhibits intestinal tumorigenesis in chemically-induced rodent models (2); however, several recent studies indicate that aspirin use may not be associated with a reduction in incidence or risk of colorectal cancer (10, 11). Similarly, aspirin was an ineffective chemopreventive agent in a chemically-induced mouse model of urinary bladder cancer, while other NSAIDs, such as ketaprofen and sulindac, significantly decreased the incidence of transitional cell carcinoma of the urinary bladder by > 70% in these animals (17).

The results presented in this paper demonstrate that while both aspirin and indomethacin decreased eicosanoid biosynthesis in the intestine, only indomethacin reduced the tumor load in these animals. We observed that tumor number and size were not significantly different in two independent experiments with aspirin used at a dose of 1.2 mg/day for these male mice, but that basal and *ex vivo* production of $PGE_2$ and 6-keto-$PGF_{1\alpha}$ were significantly reduced by 77-88%. A clear explanation for these seemingly dichotomous results is complex if one assumes that NSAIDs reduce tumor load by inhibiting prostaglandin biosynthesis. Other NSAIDs such as sulindac (4, 6) and piroxicam (5) are as efficacious as indomethacin in reducing tumor load in the *Min*/+ mouse model, suggesting that aspirin may not be as effective in reducing tumor load as other NSAIDs. Our results contrast a recent paper by Barnes and Lee who reported that treatment of *Min*/+ mice with aspirin at a dose of 1.3 mg/day and 1.2 mg/day for males and females, respectively, resulted in a 55% reduction in tumor number, but had no effect

on intestinal mucosa $PGE_2$ production (15). The contrasting results observed in prostaglandin production in these two studies may be explained by differences in experimental and analytical methodologies. The diets were different, tissue preparations for eicosanoid measurements were dramatically different, analytical procedures were different and expression of the data was different.

Aspirin's inability to reduce tumor load is perplexing since the commonly accepted hypothesis for the antitumor action of NSAIDs centers around their ability to reduce prostaglandin biosynthesis. The efficacies of aspirin and indomethacin may be related to their mode of action in inhibiting COX-2 (as reviewed in Ref. 9). Indomethacin is a slow irreversible inhibitor of both COX-1 and COX-2 by binding tightly to the active sites of the enzymes. Acetylation of the active sites by aspirin irreversibly inhibits COX-1 activity and modifies the product profile of COX-2, from $PGG_2$ to15(R)-HETE. However, the kinetic differences between indomethacin and aspirin for the inhibition of COX-2 activity may provide insight as to their abilities to reduce tumor load. The relative potencies of aspirin and indomethacin to inhibit COX-1 and COX-2 using purified enzymes, broken cell preparations and intact cells are similar, but the $IC_{50}$ values for aspirin on COX-2 activity are >100 times higher than those of indomethacin (18). However, differences in the ability to inhibit COX-2 may only provide a partial explanation. Administration of the selective COX-2 inhibitors nimesulide and MF-tricyclic reduced intestinal tumor number by 50% in *Min*/+ mice (19) and 60% in $APC^{\Delta 716}$ knockout mice (20), respectively, significantly less than the 85-95% reductions observed following treatment with indomethacin, piroxicam or sulindac, which are preferential inhibitors of COX-1 (5, 6). Even cross-breeding

*APC*$^{\Delta 716}$ knockout mice with COX-2 knockout mice failed to eliminate all intestinal tumors (20), suggesting that COX-2 activity is an important, but partial determinant of intestinal tumor load and that multiple mechanisms may contribute to the chemopreventive efficacy of NSAIDs. Taken together, these results suggest that aspirin may not be as effective in reducing tumor load in the *Min*/+ mouse model when compared to other NSAIDs, such as indomethacin, and that inhibition of prostaglandin production may not fully explain their antitumor effects.

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# PART V

# SUMMARY AND CONCLUSION

The commonly accepted hypothesis of the antitumor effect of NSAIDs is directly related to their ability to reduce eicosanoid biosynthesis by inhibiting cyclooxygenase activity. To address this hypothesis we set out to establish a positive relationship between eicosanoid biosynthesis, in particular prostaglandin $E_2$, and intestinal tumor load in the *Min/+* mouse model. We used this model because most human intestinal cancers have an underlying defect in the *APC* gene and *Min/+* mice are genotypically and phenotypically similar to human FAP (Table 2.2). *Min/+* mice were treated with different NSAIDs-sulindac, aspirin and indomethacin, each with a distinct ability to inhibit cyclooxygenase activity, to curtail and dietary arachidonic acid to augment eicosanoid biosynthesis.

Our data demonstrated that sulindac and indomethacin are very potent chemotherapeutic agents in which they significantly reduced the tumor load by ~95% and ~85%, respectively. However, aspirin, the most commonly used NSAID and a potent cyclooxygenase inhibitor, had no effect on reducing tumor load. Both aspirin and indomethacin significantly reduced basal and *ex vivo* levels of $PGE_2$ and 6-keto-$PGF_{1\alpha}$; however, administration of sulindac failed to reduce eicosanoid production in intestinal tissues. In addition, increasing overall eicosanoid biosynthesis with dietary arachidonic acid supplementation had no effect on tumor number or size; suggesting the antitumor effect of NSAIDs appears to be independent of eicosanoid biosynthesis. These results are not without precedence. For example, whereas most epidemiological studies report a 40-50% reduction in the incidence of colorectal cancer with regular aspirin use, some studies, nevertheless, report that the incidence of colorectal cancer actually increases slightly in aspirin users (1, 2). Barnes and Lee reported that aspirin, at a dose which decreased

tumor load by 50% in the *Min/+* mouse, did not inhibit prostaglandin production (3). In addition, sulindac sulfone, a metabolite of sulindac which lacks the ability to inhibit cyclooxygenase, reduced tumor load by 25% and 60% in *Min/+* mice and the AOM-induced colonic tumor rat model. Despite the fact that our data and that of others demonstrate an inconsistent relationship of eicosanoid biosynthesis and tumor load, there is sufficient evidence linking the AA cascade with intestinal cancer. For example, Ohima *et al.* (4) crossbred COX-2 knock out mice with $Apc^{\Delta 716}$ knock out mice and reduced tumor load by 86%. This data clearly demonstrated that COX-2 is involved in tumorigenesis but this study also clearly demonstrated that COX-2 is only partially responsible because not all tumors were eliminated in the COX-2 knock out mice. Overall, these data suggest that multiple regulatory mechanisms may contribute to the chemopreventive efficacy of NSAIDs.

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# APPENDIXES

## APPENDIX I

## TISSUE FATTY ACID ANALYSIS

Note: KEEP EVERYTHING AS COLD AS POSSIBLE

**Part I: Extraction of fatty acids**

1). Liver was perfused and small intestine was washed with cold saline and removed from animal for immediate process or freezing in liquid nitrogen and store at -80°C

2). Homogenize tissues in cold 0.9% saline (100mg/ml) with glass homogenizer.

3). Transfer 0.8ml of homogenates into teflon coated 16 x 125 screw top tubes and add 3ml of chloroform:methanol (1:2).

4). Vortex and incubate for 30 min. on ice in dark condition.

5). Add 200 $\mu$l saturated NaCl and 1ml of chloroform to each sample.

6). Vortex and incubate for 15 min. on ice in dark condition.

7). Centrifuge at 900xg for 3 min. (Solely for phase separation)

8). Remove chloroform layer. (bottom layer) and transfer to 12 x 75 mm test tube.

9). Extract one more time by adding 1 ml chloroform and follow the steps 6-8.

10). Evaporate the chloroform under nitrogen to dryness.

11). Resuspend the fatty acid extraction in 50 $\mu$l chloroform.

12). Separate polar fraction (phospholipids) and non-polar fraction (triglyceride, cholesterol, and free fatty acids) with TLC (Thin Layer Chromatography) by mobile phase: chloroform:methanol (8:1).
Place filter paper in the TLC chamber and pour mobile solvent **$CHCl_3$:MeOH (8:1)** into chamber

For liver: apply 25 $\mu$l to TLC silica gel 60 HP-TLC plates
For small intestine: apply all 50 $\mu$l to TLC silica gel 60 HP-TLC plate

Add additional 200 $\mu$l chloroform to each tube and flush with nitrogen and store at -20°C.

13). Place TLC plate into chamber and run TLC for about 15 min. or until solvent reach the top line. Dry TLC plate under hood.

**Part II: Saponification, acidification, and methylation of fatty acids**

14). Phospholipid fraction is scraped from point of origin of plate using razor blade and placed in a 16 x 125 mm screw top tube (make sure the tops fit tightly).

15). Add 100 $\mu$l 15:0 (1mg/ml in iso-octane) to the screw top tubes as internal standard.

16). Add 0.25 ml toluene (for solubilization)

17). Saponify the fatty acids with 0.5 ml 0.5 N KOH/MeOH, cap the tube tightly and incubate at 86°C water bath for 8 min.

18). Cool tubes to room temperature.

19). Acidify with 1 ml of 0.7N HCl/MeOH.

20). Extract fatty acids with 2 ml of Hexane, vortex well, centrifuge at 900 x g for 3 min. and transfer top layer to 12 x 75 mm tubes.

21). Repeat steps 19-20 1X and evaporate to dryness under nitrogen gas.

22). Resuspend lipids in 200 $\mu$l Hexane and transfer to V-vial and evaporate under nitrogen. Add additional 200 $\mu$l Hexane to tube and repeat transfer and evaporate 1X.

23). Under the hood, add 150-200 $\mu$l ethereal diazomethane and incubate 10 min. (put the caps loosely on immediately after ) for fatty acid methylation. (Bubble air will be seen)

24). Evaporate to dryness under the hood.

25). Resuspend methylated lipids in hexane, cap and vortex.
<u>For liver:</u> Bring up to 100 $\mu$l
<u>For small intestine:</u> Bring up to 10 $\mu$l

26). Analyze via gas chromatograph.

## APPENDIX II

## TISSUE EICOSANOID ANALYSIS by RADIOIMMUNOASSAY (RIA)

### Part I. Tissue (small intestine) Eicosanoid Extraction

### A. *Ex vivo* eicosanoid extraction:

Weight tissue and record

1. Homogenize tissue in 1200 $\mu$l of cold Tris-HCl (0.1 M, pH 7.4)
2. Transfer 100 $\mu$l to a 12 x 75 mm tube for protein Lowry assay
3. Transfer 1000 $\mu$l to a 12 x 75 mm cap-fit tube and incubate at 37$^0$C water bath for 15 min.
4. At the end of the incubation, add 250 $\mu$l of $^3$H-PGD$_2$ (0.4 $\mu$Ci / 10 ml PBSG = 10 nCi / 250 $\mu$l PBSG) as internal standard

   Take 4 $\mu$l of $^3$H-PGD$_2$ and suspend in 10 ml PBSG will generate 0.4 $\mu$Ci / 10 ml of stock $^3$H-PGD$_2$

5. Stop the reaction with 100 $\mu$l cold acid methanol solution (90% of 100% MeOH + 10% of 88% formic acid, pH 3.5, make it one day before using and store at 4$^0$C)
6. Vortex well and place on ice
7. Prepare $C_{18}$ Sep-Pak cartridge (rinse with the order of water, MeOH , water)
8. Pour samples through Sep-Pak
9. Using 10% MeOH-formic acid to rinse tube and pass through Sep-Pak
10. Completely dry cartridge
11. Collect elute with 2 ml 100% MeOH twice into a 12 x 75 tube
12. Evaporate MeOH under nitrogen
13. Add 250 $\mu$l PBSG, vortex well
14. Take 50 $\mu$l for internal standard recovery test by sintillation counting
15. Sample is ready for RIA assay

### B. Basal eicosanoid extraction:

1. Weight tissue and record
2. Homogenize tissue in 1120 $\mu$l of cold Tris-HCl (0.1 M, pH 7.4) with 80 $\mu$l of indomethacin (stock conc. 15 mM), a COX inhibitor (final conc. 1 mM)
3. Transfer 100 $\mu$l to a 12 x 75 mm tube for protein Lowry assay
4. Transfer 1000 $\mu$l to a 12 x 75 mm cap-fit tube
5. Add 250 $\mu$l of $^3$H-PGD$_2$ (0.4 $\mu$Ci / 10 ml = 10 nCi / 250 $\mu$l) as internal standard

   Take 4 $\mu$l of $^3$H-PGD$_2$ and suspend in 10 ml PBSG will generate 0.4 $\mu$Ci / 10 ml of stock $^3$H-PGD$_2$

6. Stop the reaction with 100 $\mu$l cold acid methanol solution (90% of 100% MeOH + 10% of 88% formic acid, pH 3.5, make it one day before using and store at 4$^0$C)
7. Vortex well and place on ice
8. Prepare $C_{18}$ Sep-Pak cartridge (rinse with the order of water, MeOH , water)
9. Pour samples through Sep-Pak
10. Using 10% MeOH-formic acid to rinse tube and pass through Sep-Pak
11. Completely dry cartridge
12. Collect elute with 2 ml 100% MeOH twice into a 12 x 75 tube
13. Evaporate MeOH under nitrogen
14. Add 250 $\mu$l PBSG, vortex well
15. Take 50 $\mu$l for internal standard recovery test by sintillation counting
16. Sample is ready for RIA assay

**Part II. Radioimmunoassay (RIA)**

**A. Reagent Preparation**

1) **PBSG buffer**: (Keep at 4$^0$C approximately one week)
0.1M pH 7.4 phosphate buffered saline (0.9%) with gelatin (0.1%)

(based on 500 ml formula)
2.7 grams $NaH_2PO_4.H_2O$ Sodium Phosphate Monobasic
8.2 grams $Na_2HPO_4.7H_2O$ Sodium Phosphate Dibasic (Disodium Hydrogen Phosphate)
4.5 grams NaCl
0.5 grams gelatin
Predissolve gelatin in 100ml water and heat at 50-70 $^0$C (122 -158 $^0$F) prior to addition to the buffer

Bring volume up to 450 total (pH ~ 6.825)
Adjust to pH 7.4 with NaOH
Q.S. to 500ml with distilled water

2) **Antiserum (Ab)**: (Advance Magnetics)
Reconstitute the antiserum with amount of the PBSG buffer as instructed on the label and store at -20 $^0$C
* Antiserums are packed under vacuum, need precaution when reconstitute
- Inject 2 ml of PGSB into the antiserum bottle with Hamilton syringe to wet the powder antiserum and avoid explosion of antiserum to the air when the cap is opened
- Inject air into the bottle to decrease the vacuum
- When the vacuum is low, open the rubber cap carefully, and add the rest volume of required PBSG into the bottle

3) **Tracer Preparation $^3$H-Antigens (*Ag)** (New England Nuclear)

Stock solution for:

$^3$H-$PGE_2$ contains 18,000 cpm/0.1 ml PBSG
$^3$H-6-keto-$PGF_{1\alpha}$ contains 12,000 cpm/0.1ml PBSG
$^3$H-$LTB_4$ contains 18,000 cpm/0.1 ml PBSG
Store at -20 $^0$C

4) **Standard Eicosanoid Preparation (Ag)**

1. Eicosanoid standards come with 1 mg powder
2. Dilute with 1 ml MEOH; the concentration will be 1 mg/ml MEOH
3. Taking 10 $\mu$l eicosanoid-MEOH solution (1 mg/ml), evaporating MEOH, diluting with 1 ml MEOH again. The concentration will be 10 $\mu$g/ml = 10,000 ng/ml MEOH (stock solution)
4. To prepare working solution, evaporate 10$\mu$l of stock solution (100 ng/10 $\mu$l) and redissolve in 1ml PBSG buffer, the concentration will be (100 ng/ml PBSG) Store at -20 C.

Dilute working solution (WS) with PBSG buffer as follows:

| Stds Tubes | Conc. (pg/0.1 ml) | | Log Conc. |
|---|---|---|---|
| S-1 | 2000 | 0.2 ml WS plus 0.8 ml PBSG | 3.30 |
| S-2 | 667 | 0.5 ml of S-1 plus 1.0 ml PBSG | 2.82 |
| S-3 | 222 | 0.5 ml of S-2 plus 1.0 ml PBSG | 2.35 |
| S-4 | 74 | 0.5 ml of S-3 plus 1.0 ml PBSG | 1.87 |
| S-5 | 24.7 | 0.5 ml of S-4 plus 1.0 ml PBSG | 1.39 |
| S-6 | 8.2 | 0.5 ml of S-5 plus 1.0 ml PBSG | 0.91 |
| S-7 | 2.7 | 0.5 ml of S-6 plus 1.0 ml PBSG | 0.43 |

Use standard tubes (S-1 - S-7) to generate the standard curve
Prepare standards shortly before use

5) **Dextran-Coated Charcoal (DCC)**

2.5g Activated Charcoal
300mg Dextran
250 ml PBSG buffer

The charcoal suspension is heated at 50-60 $^0$C (122-140 $^0$F) for 30 min. with stirring, then chilled in ice water and stored at 4 $^0$C. (Prepare DCC one day before assay is to be run.)

| In 125ml PBSG buffer, add: | In 65 ml PBSG buffer, add: |
|---|---|
| 1.25g Charcoal | 0.65g Charcoal |
| 150mg (0.15 g)Dextran | 75mg (0.075 g) Dextran |

| In 250ml PBSG buffer, add: | In 500ml PBSG buffer, add: |
|---|---|
| 2.5g Charcoal | 5.0g Charcoal |
| 300mg Dextran | 600mg Dextran |

**B. Assay Protocol**

1). Thaw all frozen reagents and samples completely
2). Number a set of 12 x 75mm tubes as follows:

| Tube No. | Buffer | Std. or sample (Ag) | Tracer (*Ag) | Antiserum (Ab) | DCC |
|---|---|---|---|---|---|
| 1 Total Counts | 950$\mu$l | 0 | 100$\mu$l | 0 | 0 |
| 2 NSB (blank) | 200$\mu$l | 0 | 100$\mu$l | 0 | 750$\mu$l |
| 3 Bo | 100$\mu$l | 0 | 100$\mu$l | 100$\mu$l | 750$\mu$l |
| 4-10 Stds. | 0 | 100$\mu$l | 100$\mu$l | 100$\mu$l | 750$\mu$l |
| 11- Samples | 0 | 100$\mu$l | 100$\mu$l | 100$\mu$l | 750$\mu$l |

** Total count----no binding
NSB---non specific binding 1-2% of TC
Bo----Total binding 40-50% of TC

**NOTE**: To determine how much volume of sample to use--> make a set of dilutions, i.e. 1:5, 1:25, 1:50, 1:100. Run these diluted samples and choose those the data points fall on the linear portion of the standard curve.

3). Add 950$\mu$l PBSG buffer to tube 1
4). Add 200$\mu$l PBSG buffer to tube 2
5). Add 100$\mu$l PBSG buffer to tube 3
6). Add 100$\mu$l of standards to tube 4-10, starting with the lowest concentration level of standard solution
7). Add 100$\mu$l of sample starting in tube 11
8). Add 100$\mu$l of tracer to each tube
9). **Starting with tube 3**, add 100$\mu$l of antiserum to each tube
10). Vortex and incubate for 1 hr. at room temp and overnight at 4 $^0$C

11). At least 15 min. before the end of the overnight incubation period, place the DCC in an ice bath and stir continuously at moderate speed
12). **Except for tube 1**, all tubes add 750$\mu$l DCC each and vortex for 3-5 seconds
13). Centrifuge 20 min. at 1000 x *g* and at 4 $^0$C (except tube 1)
14). Without disturbing the sediment, decant all of the supernatant from each tube and place in scintillation vials (avoid charcoal). Add 10ml scintillation fluid and count on the scintillation counter
15). Calculate the percent **B/Bo**

$$\frac{\text{(cpm of std or sample - cpm of NSB)}}{\text{(cpm of Bo-cpm of NSB)}} * 100$$

16). Plot standard curve: B/Bo against Log conc.
* NSB should be 1-2% of the Total count.
* Bo should be 40-50% of the Total count.

## C. RIA principle

1. Preparation of Standard curve:
- fixed amount of Ab (antiserum) and *Ag ($^3$H-eicosanoid)
- known but increasing amounts of cold Ag (standard eicosanoid)

2. Principle:

I. Ab + *Ag ----> Ab-*Ag + increasing Ag ----> Ab-Ag + Ab-*Ag + Ag + *Ag

II. To remove extra Ag and *Ag by adding activated charcoal with dextran coated.

The charcoal is coated with cross-linked polysacchoride dextran. The activated charcoal has very small pore sizes and can bind small molecules instantaneously, but since they are dextran coated, only the smaller (non-complexed molecules) can penetrate to bind. Therefore Ab-Ag complex will not be bound to the activated charcoal.

III. Total counts- add no charcoal, Ag or Ab.

Non-specific binding (NSB) - to ensure all the free *Ag binds to the activated charcoal.

*should be 1-2% of the total counts.

Total binding (Bo) - to make sure the binding sites of Ab are saturated by *Ag. should be 40-50% of total concentration.

## APPENDIX III

## LOWRY PROTEIN ASSAY

### Modified Lowry Protocol

I. Reagents:

1). Buffer A:

| | | |
|---|---|---|
| 2.0% $Na_2CO_3$ | 20 g/l | 10 g/500 ml |
| 0.4% NaOH | 4 g/l | 2 g/500 ml |
| 0.16% Sodium Tartrate | 1.6 g/l | 0.8 g/500 ml |
| 1.0% SDS | 10 g/l | 5 g/500 ml |

2). Buffer B:

| | | |
|---|---|---|
| 4.0% $CuSO_4*5H_2O$ | 40 g/l | 20 g/500 ml |

3). Buffer C:

Mix 99 parts buffer A with 1 part buffer B to make alkaline copper reagent.

| Total (ml) | A (ml) | B (ml) | Assays |
|---|---|---|---|
| 50 | 49.5 | 0.5 | 14 |
| 100 | 99 | 1 | 30 |
| 150 | 148.5 | 1.5 | 48 |
| 200 | 198 | 2 | 64 |

4). Buffer D:

Dilute Folin-Ciocalteu 2 N phenol reagent, 1:1 (v/v) with deionized $H_2O$.

5). Buffer E (prepare fresh):

0.01% (=0.1mg/ml) BSA in deionized $H_2O$

II. Sample Preparation:

Tissues are homogenized in saline and frozen at -20 $^0$C

After thawing, sample is sonicated to disperse contents and then diluted 1:5 (may be varied from tissue to tissue)

Dilute sample one more time (1:1) with deionized $H_2O$

III. Standard Curve Preparation (duplicate):

| | Buffer E 0.01% BSA (0.1 $\mu$g/$\mu$l) | | | 0.9% saline |
|---|---|---|---|---|
| Std 1 | 0 $\mu$l | ( 0 $\mu$g) | + | 1000 $\mu$l |
| Std 2 | 100 $\mu$l | (10 $\mu$g) | + | 900 $\mu$l |
| Std 3 | 200 $\mu$l | (20 $\mu$g) | + | 800 $\mu$l |
| Std 4 | 400 $\mu$l | (40 $\mu$g) | + | 600 $\mu$l |
| Std 5 | 600 $\mu$l | (60 $\mu$g) | + | 400 $\mu$l |
| Std 6 | 800 $\mu$l | (80 $\mu$g) | + | 200 $\mu$l |
| Std 7 | 1000 $\mu$l | (100 $\mu$g) | + | 0 $\mu$l |

IV. Assay procedure:

1). To 50 $\mu$l of sample add 1ml of saline (0.9%), duplicate
2). Add 3ml of reagent C to each sample and standards. Incubate at room temperature for 15 min.
3). Add 300 $\mu$l of diluted (1:1) Folin-Ciocalteau (reagent D) to each sample and standards; vortex vigorously
4). Incubate for 45 min. at room temperature and read at 660nm against the blank.

V. Reference:

Lowry, O.H. Rosebrough, N.J., Farr, A.C. and Rondall Ross J.J. Biol. Chem. 193 (1951):265.

## APPENDIX IV

# RELATIONSHIP OF β-CATENIN AND Bcl-2 EXPRESSION TO SULINDAC INDUCED REGRESSION OF INTESTINAL TUMORS IN *MIN*/+ MICE

Michael F. McEntee, Chun-Hung Chiu, Jay Whelan

This manuscript has been submitted in similar format to Cancer Research

## ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) can cause regression of intestinal tumors in humans and laboratory rodents, but relatively little is known about the mechanisms involved. Although cyclooxygenase-2 appears to have a role in tumor growth, it is not yet clear whether blocking prostaglandin synthesis entirely accounts for the anti-tumor activity of NSAIDs. Previous work has examined the affects of various manipulations on tumor load in order to deduce mechanistic conclusions without examining the regressing tumors themselves. We recently showed that the NSAID sulindac rapidly decreases the small intestinal tumor load in Min mice but does not appear to have the same impact on colonic lesions. In the present study, we have directly examined tumors harvested from Min mice during sulindac-induced regression, and after prolonged treatment (20 days) at which point the tumor load has stabilized. Using immunohistochemistry with morphometrics (for β-catenin) and a scoring scheme (for Bcl-2), we found a ≥50% decrease in β-catenin ($p=0.001$) and diminishing Bcl-2 ($p=0.019$) in small intestinal tumors undergoing sulindac-induced regression when compared to those from untreated animals. In contrast, β-catenin and Bcl-2

staining in small intestinal tumors from animals treated for 20 days was not significantly different from untreated controls. There was also no significant difference in staining for either protein in any of the colonic tumors, regardless of treatment status. These findings suggest that modulation of β-catenin may occur during NSAID-induced regression of intestinal adenomas, and that Bcl-2 may confer resistance to these effects.

## INTRODUCTION

Development of colorectal cancer, from the appearance of benign adenomatous polyps to metastatic carcinoma, involves a series of genetic mutations ("hits"), the earliest often involving the *APC* gene (1, 2). Individuals affected with familial adenomatous polyposis (FAP) carry a germline mutation in *APC* and mutational damage or loss of the second wild type allele initiates intestinal tumor formation (3, 4). Somatic mutations resulting in loss of full length APC protein also occur early in spontaneous forms of the disease (1). The protein is normally up-regulated as cells exit small intestinal crypts or near the luminal surface of the colon and is believed to have a role in controlling cell migration, apoptosis, and possibly proliferation (5). An important molecular function of APC is regulation of cytoplasmic pools of β-catenin. β-Catenin is a necessary component of the E-cadherin adhesion complex and a transduction molecule for the Wnt-1 (Wingless in Drosophila) signaling cascade, cooperating with at least one other factor (Lef-1/TCF) in transcriptional regulation of as yet incompletely described genes (6-9). APC contributes to a degradation process that eliminates cytoplasmic β-catenin molecules not bound to E-cadherin and thereby down-regulates β-catenin signaling (5). Although the biologic role of this signaling cascade in mammals is not

fully understood, loss of APC function results in increased levels of free β-catenin in the cytoplasm and nucleus where it presumably effects some aspect of cell behavior (10). Clinical studies indicate that non-steroidal anti-inflammatory drugs (NSAIDs) cause regression of pre-existing adenomas in FAP patients (11) but the mechanism(s) is still a matter of speculation. One probable affect of NSAIDs on these tumors is induction of apoptosis (12-17). Bcl-2 is a key regulatory antagonist of apoptosis and it has been shown that over-expression of this protein commonly occurs relatively early in the progression of colorectal carcinogenesis (18-20).

The Min mouse model has been used by a number of laboratories to investigate the affects of NSAIDs on intestinal tumor biology (14, 21, 22). As in humans with FAP, Min mice carry a germline mutation in *APC* and develop multiple intestinal adenomas following spontaneous loss of the wild type allele (23). In a recent report, we showed that the NSAID sulindac causes very rapid regression of pre-existing tumors in Min mice and is necessary for sustained control of tumor numbers in the intestinal tract (24). In the present study we have looked at the distribution of β-catenin, APC and Bcl-2 in Min mouse tumors before and after sulindac treatment in an effort to correlate molecular changes with tumor behavior and responsiveness to NSAIDs.

## METHODS

Tissue samples were obtained during the course of a previously reported study and details of the experimental protocol are presented therein (24). In brief, heterozygous *Min/+* mice (Min) were obtained from Jackson Laboratories (Bar Harbor, ME) at approximately 35

days of age and immediately started on a powder diet (AIN-93G; Dyets, Inc., Bethlehem, PA). Sulindac (Sigma Chemical Co., St. Louis, MO) was added to the diet at 320 ppm for the treatment groups. Mice were randomly assigned to 4 different groups:

Controls (no sulindac supplementation) sacrificed at 80 days of age.

Sulindac starting at 78 days of age, sacrificed at 80 days (2 days on sulindac).

Sulindac starting at 78 days of age, sacrificed at 82 days (4 days on sulindac).

Sulindac starting at 78 days of age, sacrificed at 98 days (20 days on sulindac).

All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accord with the NIH *Guide for the Care and Use of Laboratory Animals* (NRC 1985). At sacrifice, the entire intestinal tract was removed, flushed with cold ($4^0$C) phosphate buffered saline (PBS) and opened longitudinally. Tissue samples were harvested under a dissecting microscope and fixed in 10% neutral buffered formalin. Four μm paraffin embedded sections were placed on Probe-ON Plus slides (Fisher Scientific, Pittsburgh, PA ) and immunohistochemically stained using the standard indirect peroxidase biotin-streptavidin technique. Sections to be stained for Bcl-2 were pretreated with a microwave antigen retrieval procedure using 10 mM citrate buffer at pH 6 (10 minutes). All sections were pre-blocked with 3% $H_2O_2$ in PBS (15 minutes) and normal goat serum blocking reagent (50 minutes; BioGenex, San Ramon, CA). Sections were incubated for 1 hour at room temperature with antibodies to β-catenin (cat. #C19220, 0.5 μg/ml; Transduction Laboratories, Lexington, KY), or the carboxyl-terminus of APC (cat. #sc-896; 2.5 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), or overnight at $4^0$C with rabbit anti-mouse Bcl-2 (cat. #15616E, 1:300; PharMingen, San Diego, CA). Sections were then

incubated with biotinylated anti-mouse or anti-rabbit immunoglobulin (50 minutes, room temperature) followed by streptavidin-peroxidase link (40 minutes, room temperature; BioGenex). Localized peroxidase conjugates were visualized with 3,3'-diaminobenzidine (DAB; BioGenex) and sections were lightly counter stained with hematoxylin. Internal positive controls included normal mucosal epithelial cells or lymphocytes (for Bcl-2). Primary antibody was eliminated or an irrelevant antibody substituted in its place as negative controls for each set. For APC, additional controls included preincubation (overnight at $4^0$C) of 1 μg antibody with 11 μg APC peptide (cat #sc-896P; Santa Cruz Biotechnology) which eliminated positive staining.

Staining was analyzed subjectively and, for β-catenin, objectively by morphometry. All evaluations were done on coded slides without knowledge of the source of the tumor. Subjective evaluation entailed direct comparison of staining in the tumors to components of the adjacent normal intestinal mucosa. For Bcl-2, a scoring scheme was based on comparison to normal crypt epithelial cells in immediately adjacent areas of small intestinal or colonic mucosa: no staining = 0; a staining gradient from positive deep in the tumor (at level of adjacent crypts) to negative in upper regions = 1 (i.e. "normal" distribution pattern); staining in both the deeper and upper regions of the tumor (above the level of the adjacent crypts) that was equal to or greater than that of the crypts focally/multifocally = 2, and diffusely = 3. For APC, tumor staining was compared to epithelial cells of the adjacent small intestinal villi and surface of the colon which are normally positive for this antigen (25, 26).

All morphometric measurements on β-catenin stained sections were done at a fixed magnification (4X objective) and illumination using BioQuant/TCW Image Analysis software,

version 2.2 (R&M Biometrics, Nashville, TN). Tumors were manually outlined on the screen to give an area expressed in pixels. Abnormal β-catenin staining was measured by empirically adjusting the color "threshold" setting for a typical untreated small intestinal mass to demarcate only regions with increased cytoplasmic/nuclear staining; all other tumors were then evaluated at the same setting. Demarcated areas within each tumor were summed to give a total area stained (in pixels) within the outlined tumor mass. β-Catenin data represent the percentage of each tumor to over-express β-catenin in abnormal intracellular locations as determined by a uniformly applied protocol of tissue handling, staining, and analysis.

All data were tested for normality. β-Catenin data was log transformed and analyzed by analysis of variance (ANOVA) using JMP software (SAS Institute Inc., Cary, North Carolina). Dunnett's pairwise comparison test was used to determine differences ($p<0.05$) between the experimental groups and untreated controls. The Bcl-2 data consisted of discrete numeric values and could not be normalized. Bcl-2 data was therefore analyzed using a Kruskal-Wallis non-parametric test for individual pairwise comparisons, followed by a Bonferroni's adjustment for significance. Spearman's correlation and Kendall's tau-b tests were applied to the Bcl-2 data for analysis of trend and significance was established at $p<0.05$.

## RESULTS

### β-Catenin

Normal mucosal epithelial cells of the small intestine and colon were variably, often weakly, positive under the described staining protocol; more uniform and darker staining

could be achieved by increasing the concentration of anti-β-catenin antibody or the length of its incubation with the tissues. Staining was always restricted to the lateral cell membranes and there was no consistent difference in reactivity between the colon and small intestine, or between the crypt and surface epithelium. The staining intensity of neoplastic cells in the small intestine and colon was distinctly increased, in comparison to adjacent normal epithelium, and concentrated in the cytoplasm and/or nucleus (Figure 1, insert) with little evidence of membrane staining. In most small intestinal tumors, the greatest staining was restricted to the deeper and lateral aspects of the mass (Figure 1). In the smallest lesions, consisting of single cystic expansions of the crypt (Figure 2) or lesions that had not yet progressed much beyond the involvement of one or two villi, staining was typically more widespread with a substantially greater percentage of positive cells than the majority of small intestinal tumors evaluated (mean = 61.8% versus 23.1%; $p< 0.0001$). In comparison to lesions of the small intestine, colonic tumors had significantly greater β-catenin staining (Table 1; $p=0.0009$) with a more uniform distribution from the base of the mass to the surface (Figure 3). The relative intensity and intracellular pattern of staining in neoplastic colonic epithelial cells was similar to that of cells forming small intestinal lesions.

As shown in Table 1, β-catenin staining decreased significantly in small intestinal tumors within 2 days of sulindac treatment ($p<0.0009$). Although fewer tumors were available for evaluation, this decrease was also evident after 4 days of sulindac treatment ($p=0.001$). In contrast, β-catenin in tumors from animals maintained on sulindac for 20 days was not significantly different from untreated controls ($p=0.782$). The pattern of staining in tumors from all treated animals resembled untreated controls in that reactivity was

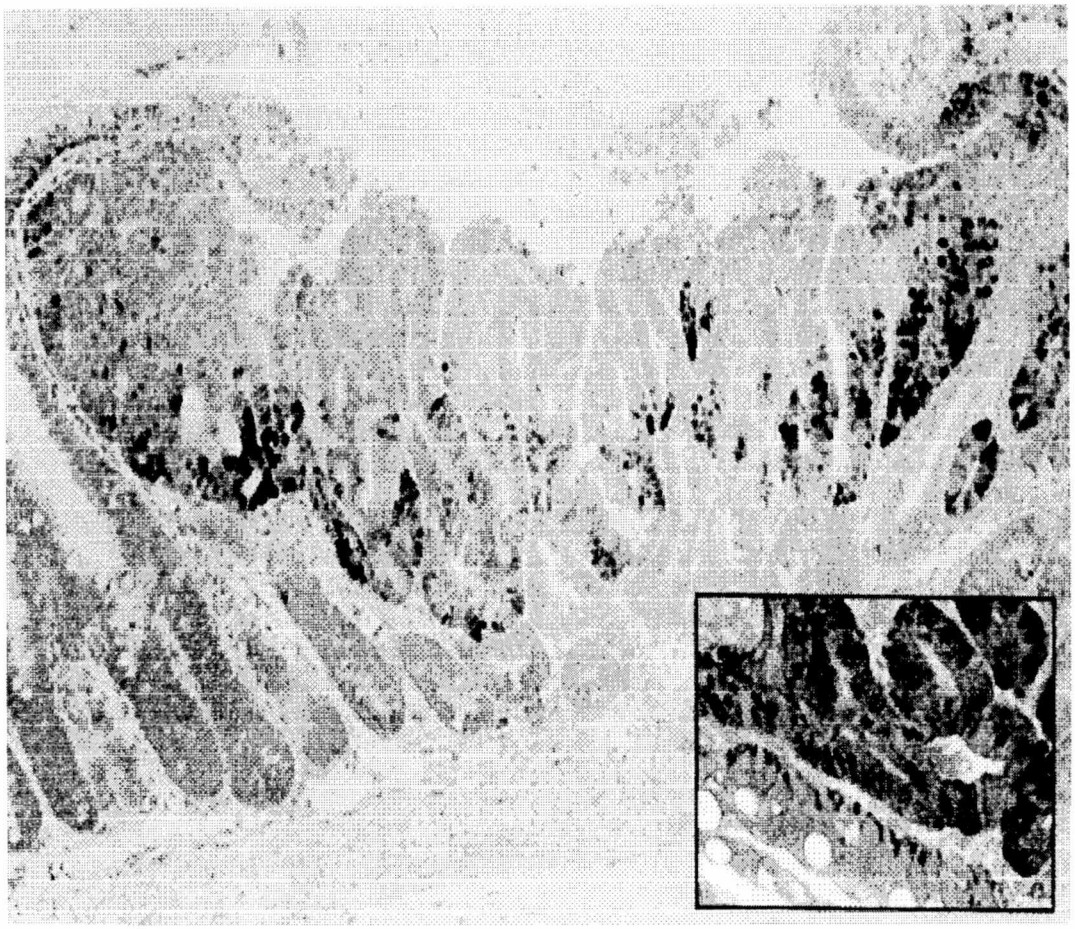

Figure A.1. Tumor from the small intestine of an untreated Min mouse, stained for β-catenin. Staining was generally most intense in deeper aspects of small intestinal tumors where the cytoplasm and nucleus of neoplastic cells were strongly reactive (insert). β-Catenin staining in adjacent normal epithelial cells was restricted to the intercellular membranes (lower left of insert).

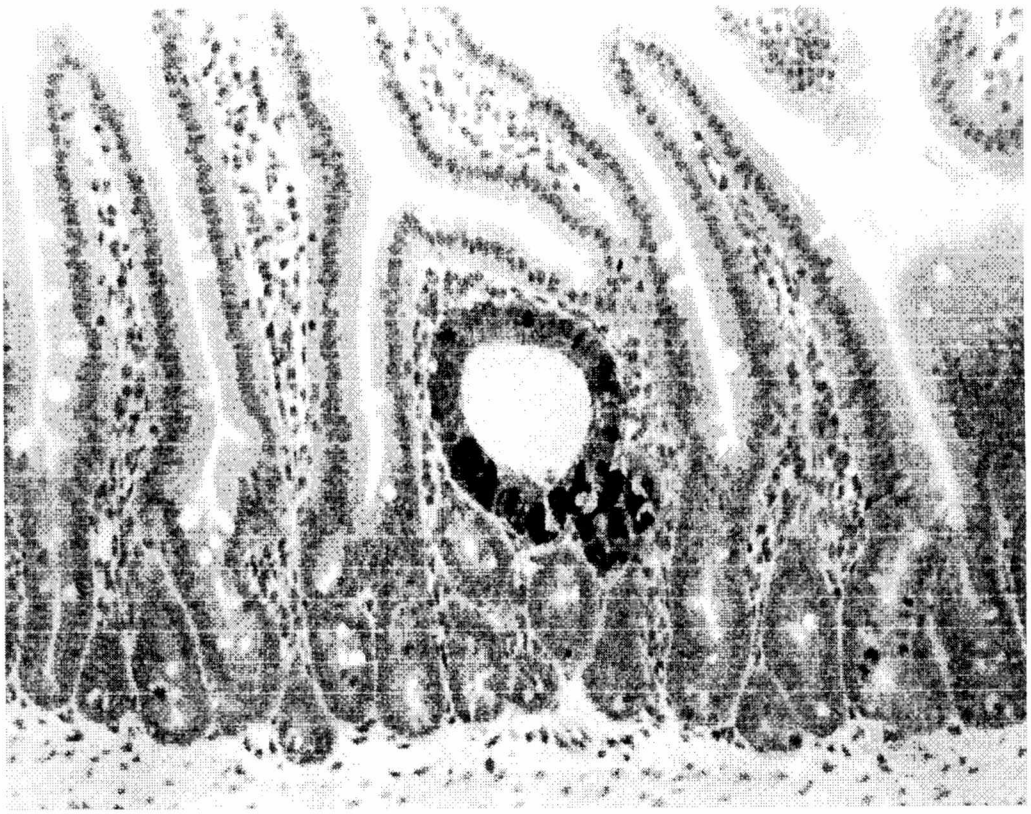

Figure A.2. Early cystic lesion from the small intestine of an untreated Min mouse with over-expression of cytoplasmic and nuclear β-catenin.

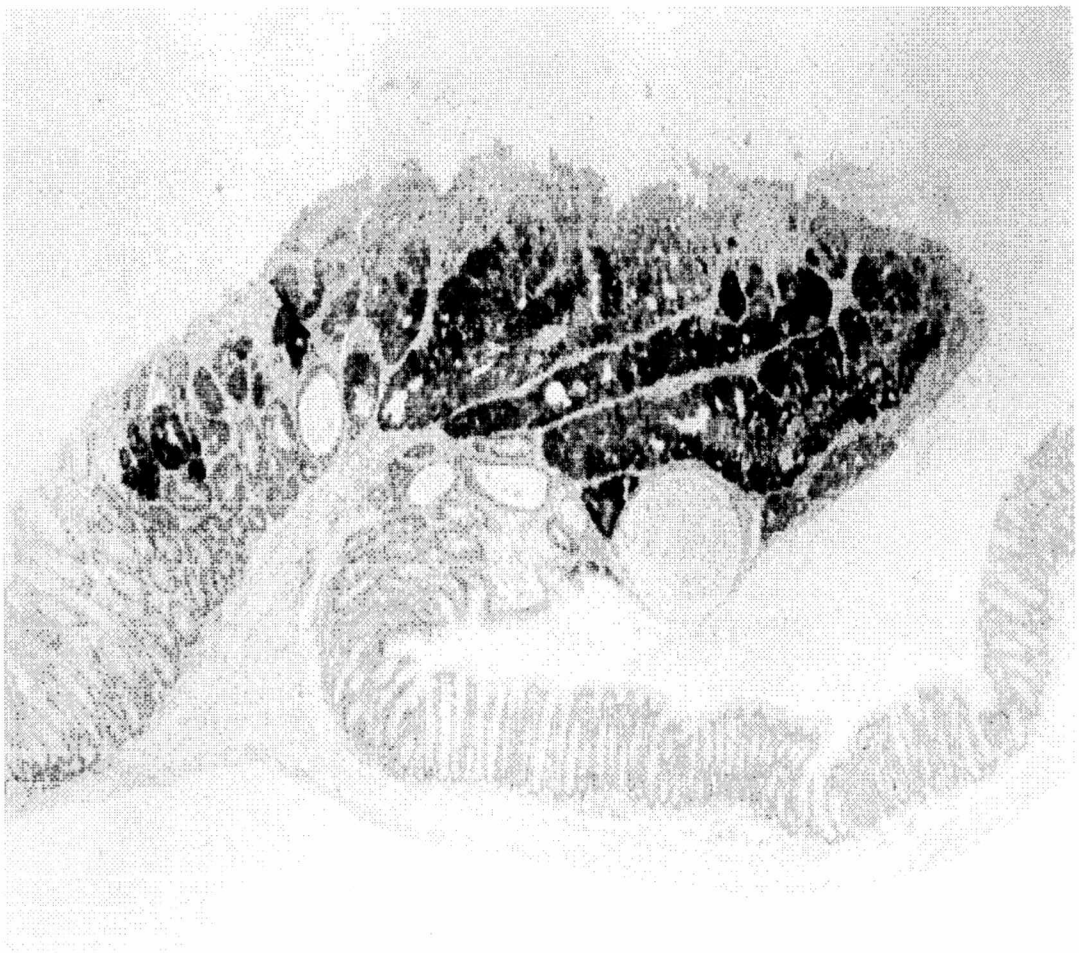

Figure A.3. Colonic tumor from untreated Min mouse stained for β-catenin.

predominantly in the deep to lateral aspects of the lesions. These cells stained in their cytoplasm and nuclei, as for untreated tumors, without an apparent increase in cytoplasmic membrane reactivity. The mean area of small intestinal tumors analyzed from all treatment groups was less than that of untreated controls and this difference was significant ($p<0.05$) for the 2 day treatment group (data not shown).

β-catenin staining in colonic tumors treated with sulindac for 2, 4 or 20 days tended to be slightly less than in untreated tumors (Table 1) but differences were not statistically significant (all p values $\geq 0.30$). There was no morphologic distinction in β-catenin staining intensity or distribution. There was also no significant difference in the size of the colonic tumors evaluated from the different treatment groups (data not shown).

**APC**

APC staining in normal small intestinal epithelial cells was greater over villi than in the crypts, where cells varied from negative to weakly positive. Reactivity in villus epithelial cells was predominantly cytoplasmic but occasionally the lateral cell membranes were delineated. A similar pattern of staining was found in the colon where deep crypt cells were negative and luminal epithelial cells were positive. Tumor cells in the small intestine and colon from untreated control animals were negative to weakly positive (comparable to that of normal crypt epithelium) and always less than that of the adjacent mature epithelium lining normal villi or the luminal colonic mucosa (not shown). There was no increase in APC staining in any of the small intestinal or colonic tumors from animals treated with sulindac for 2, 4 or 20 days.

Table A.1. Mean percent β-catenin staining and Bcl-2 scores for small intestinal (SI) and colonic tumors from untreated Min mice and those on sulindac for 2, 4 and 20 days.

| Treatment[a] | Mean % β-catenin SI Tumors (number) | Mean % β-catenin Colonic Tumors (number) | Mean Bcl-2 Score SI Tumors (number) | Mean Bcl-2 Score Colonic Tumors (number) |
|---|---|---|---|---|
| Untreated controls | 23.1[b] ±2.6 (n=50) | 60.0[b] ±9.7 (n=7) | 2.34[b] ±0.14 (n=38) | 3.0[b] ±0.0 (n=5) |
| 2 days Sulindac | 12.1[c] ±2.8 (n=39) | 54.0[b] ±9.3 (n=3) | 1.95[b] ±0.18 (n=40) | 3.0[b] ±0.0 (n=3) |
| 4 days Sulindac | 6.8[c] ±3.6 (n=6) | 42.3[b] ±2.4 (n=3) | 1.38[b] ±0.50 (n=8) | 3.0[b] ±0.0 (n=2) |
| 20 days Sulindac | 26.7[b] ±6.5 (n=18) | 40.0[b] ±11.0 (n=2) | 2.45[b] ±0.18 (n=20) | 3.0[b] ±0.0 (n=3) |

[a] Seventy-eight day old Min mice were treated for 2, 4, or 20 days with sulindac as previously described (24). Untreated controls were sacrificed at 80 days of age. β-catenin data was generated using morphometrics and indicates the relative extent of staining in individual tumors. Bcl-2 data was derived from a scoring system, also based on the amount and distribution of staining in the individual tumors, with higher numbers indicating more widespread Bcl-2.

[b] Values in each column with a different superscript letter are significantly different at $p < 0.05$. The downward trend in small intestinal Bcl-2 scores from untreated controls to the 2 and 4 day treatment groups is significant at $p=0.019$.

**Bcl-2**

Many lymphocytes in the lamina propria were positive for Bcl-2 and intraepithelial lymphocytes were often strongly reactive. The pattern of staining for Bcl-2 in normal epithelial cells of both the small intestine and colon was similar, but quantitatively distinct. In the small intestine, there was a faint homogenous to granular staining of the cytoplasm in normal crypt epithelial cells that was absent in epithelium above the crypt-villus junction (Figure 4). This reactivity for Bcl-2 was not as strong as that seen in deep crypt cells of the colon, where staining also rapidly decreased to negative in cells higher up the crypt and on the luminal surface. Neither the colonic nor small intestinal crypt epithelium stained as intensely as normal intraepithelial lymphocytes. Small intestinal tumors showed several different patterns of staining for Bcl-2. Reactivity varied from diffusely less than that of normal crypt epithelium (negative), to diffusely positive (comparable to or slightly greater than that of crypt epithelium) (Figure 5), to an intermediate pattern with staining in deeper tumor cells but none above the level of adjacent crypts (i.e. distribution similar to the normal mucosa). The mean Bcl-2 staining score for untreated small intestinal tumors was 2.34 (Table 1), with a score of 1 indicating essentially the same reactivity as the normal mucosa. Cells in some of the smallest lesions stained for Bcl-2 above the level of the adjacent crypts (Figure 4). In some serially sectioned small intestinal tumors, cytoplasmic reactivity for Bcl-2 co-localized with cytoplasmic/nuclear β-catenin. Colonic tumors were more strongly positive for Bcl-2 than their small intestinal counterparts with reactivity throughout most lesions and little evidence of a staining gradient. Staining of most colonic tumor cells was equal to or greater than that of the adjacent normal crypt epithelium and in some instances showed

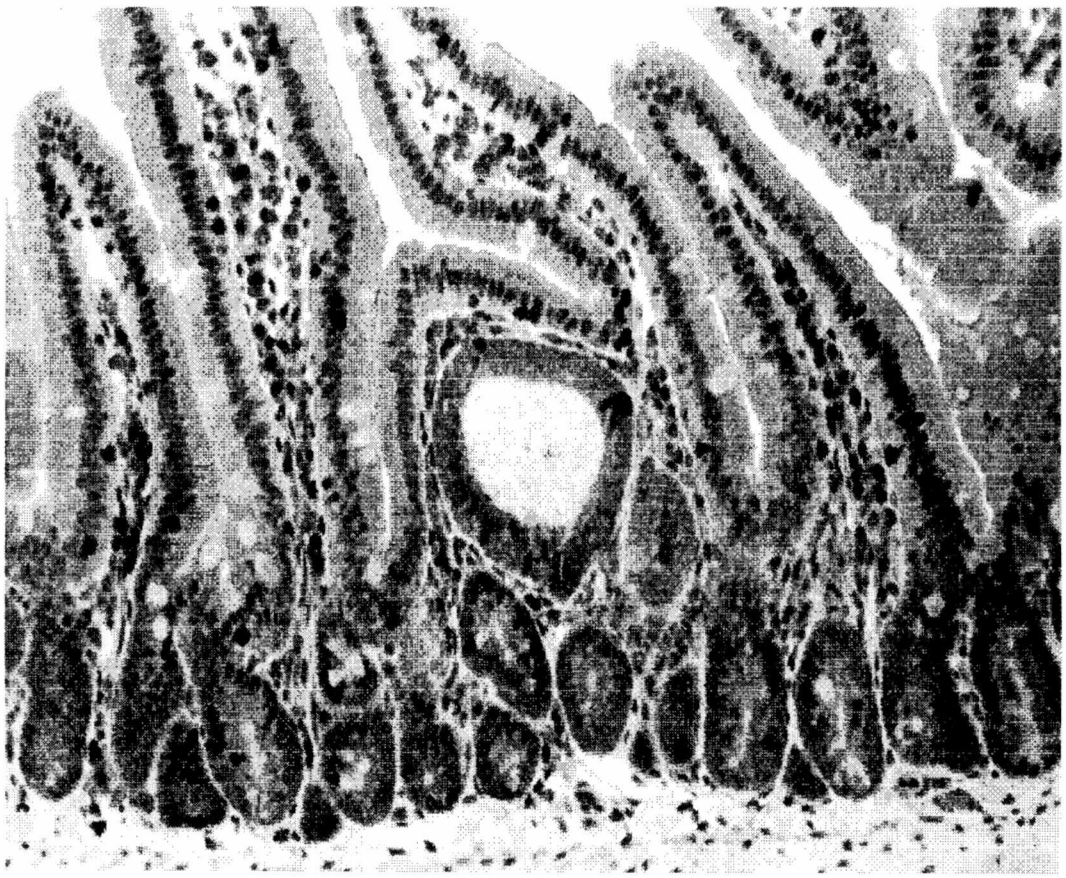

Figure A.4. Cystic lesion (same as in Figure 2) from the small intestine of an untreated Min mouse stained for Bcl-2. Crypt cells and the cystic lesion are weakly positive for Bcl-2 whereas epithelial cells above the crypt-villus junction are negative. Intraepithelial lymphocytes are strongly positive for Bcl-2 (arrow).

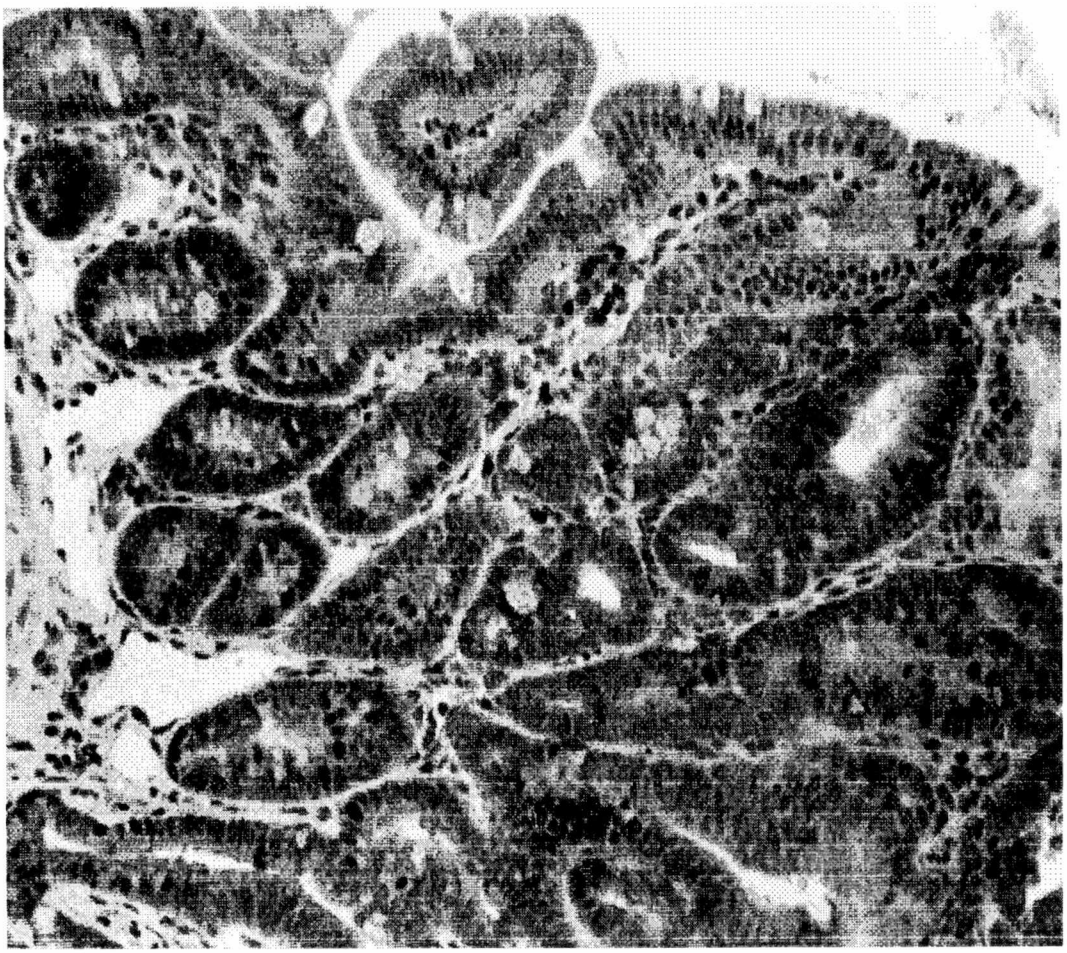

Figure A.5. Edge of small intestinal tumor from untreated Min mouse stained for Bcl-2. Most of the tumor cells are positive for Bcl-2 whereas the mature villus epithelium of the adjacent mucosa is negative.

increased reactivity around nuclear membranes (Figure 6).

In small intestinal tumors there was a sequential decrease in the mean Bcl-2 score after 2 and 4 days of sulindac treatment (Table 1). There was no difference between Bcl-2 staining in control tumors and those from animals on sulindac for 20 days ($p>0.5$). Although comparison of the 4 day group to untreated controls yielded a low p value (0.0425), this was not statistically significant after applying Bonferroni's adjustment which indicated significance only at $p<0.017$. However, the trend in Bcl-2 scores to decrease from untreated controls to the 2 and 4 day treatment groups was statistically significant ($p=0.019$). All colonic tumors from treated and untreated animals stained similarly for Bcl-2 and were given a score of 3.

## DISCUSSION

The earliest molecular event that typically heralds the appearance of intestinal tumors is an *APC* mutation(s) which results in loss of the full-length protein and an accumulation of β-catenin in the cytoplasm and nucleus of affected cells (1, 5, 10). We have confirmed this in Min mouse tumors and have further shown that cystic crypts in the small intestine, previously described as the earliest stage of tumor growth (27), are also strongly reactive for nuclear and cytoplasmic β-catenin. These findings are consonant with an initiating somatic mutation in *APC*, resulting in loss of heterozygosity, and the lack of immunohistochemical reactivity for the carboxyl-terminus of APC in these lesions (25, 26).

There was clearly a greater amount of β-catenin in tumor cells than in the adjacent normal mucosal epithelium. Staining in normal cells, always restricted to the lateral cell membranes, was often weak or negative under the described staining protocol whereas tumor

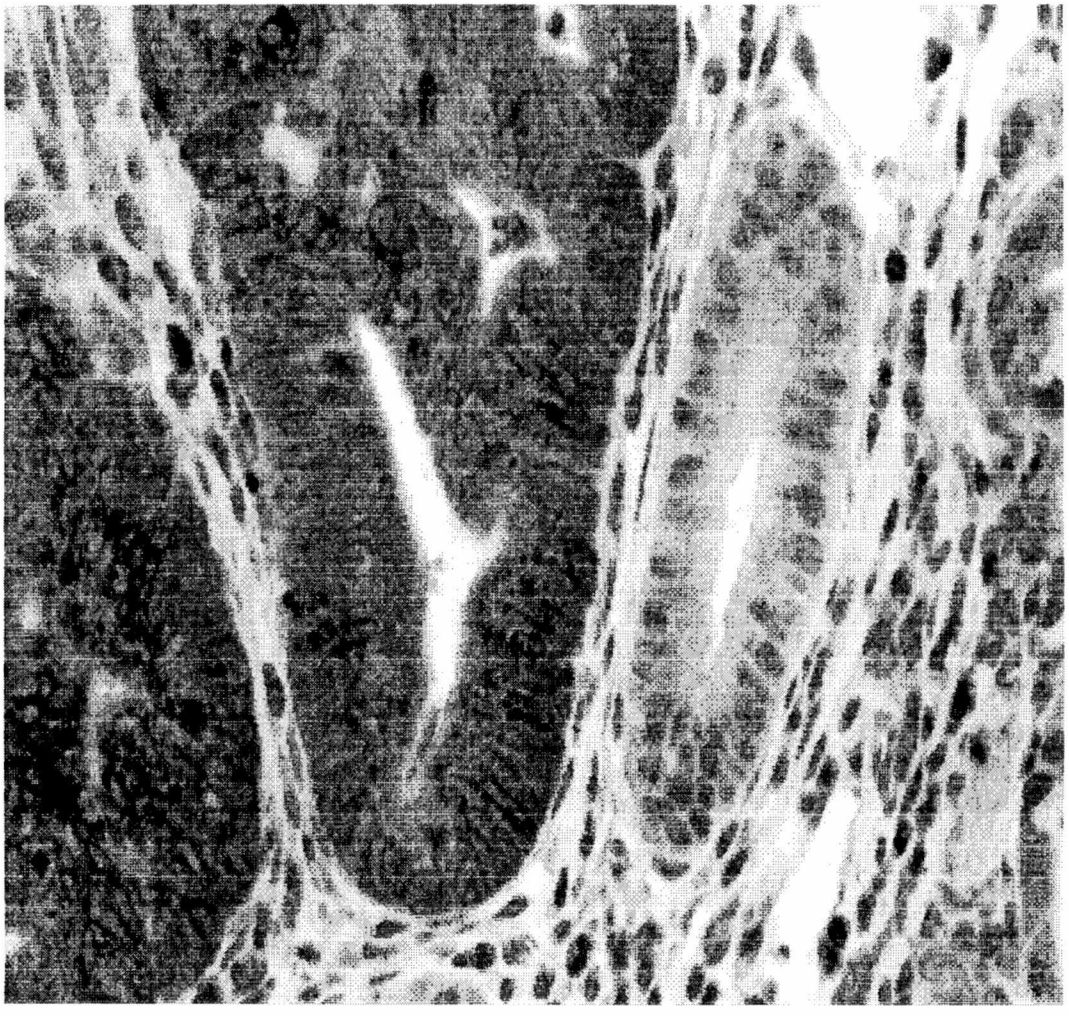

Figure A.6. Bcl-2 staining in colonic tumor cells distinguishes them from the adjacent non-neoplastic epithelium. Bcl-2 reactivity in tumor cells often outlines the nuclear envelope.

cells were darkly stained across their cytoplasm and/or nucleus. A staining gradient was evident in small intestinal tumors due to greater β-catenin reactivity in deeper aspects of these lesions. This is not believed to reflect a qualitative change in the intracellular distribution of β-catenin because increasing the concentration or incubation period of anti-β-catenin antibody in the staining protocol increased cytoplasmic/nuclear staining in the upper regions of these tumors without an increase in membrane staining (not shown). It is worth emphasizing that the staining conditions were identical for all samples and, as such, reflect the relative amount of β-catenin in various intracellular locations and in various regions of both the tumors and the normal mucosa. An explanation for this pattern of β-catenin staining in small intestinal tumors is not obvious. We did not detect a similar gradient in the normal mucosa nor in colonic tumors. Although a gradient has been previously described in the normal intestinal mucosa, concentrations increased towards the lumen rather than towards the submucosa (10, 28).

Non-steroidal anti-inflammatory drugs have been shown to impact on intestinal tumor growth and although COX-2 is apparently important in this process (14, 29, 30), evidence indicates that other mechanisms are involved which may mediate the affect(s) of NSAIDs (15, 17, 31, 32). We have shown in this study that there is a significant decrease in β-catenin in small intestinal tumors from animals actively experiencing NSAID-induced tumor regression. In a previous study these lesions were shown to undergo regression after only 2 to 4 days of sulindac treatment, with little further change in tumor load after 20 days or more of drug administration (24). β-catenin staining in small intestinal tumors from animals treated with sulindac for 2 days was approximately half that in untreated tumors, with a further

reduction after 4 days of treatment. This decrease does not involve a change in the intracellular distribution of the protein (i.e., no increase in membrane staining), but may be due to a decrease in total β-catenin levels such that fewer cells are immunohistochemically reactive. Since APC staining did not increase in tumors treated with sulindac for 2 or 4 days, the decrease in β-catenin was not attributed to repopulation with normal cells (i.e., those containing a wild-type allele). In contrast to the 2 and 4 day treatment groups, the percentage of β-catenin staining in small intestinal tumors harvested from animals on sulindac for 20 days was essentially the same as in tumors from untreated animals. Similarly, β-catenin did not decrease to a significant degree in colonic lesions from any of the treated animals. Decreased β-catenin in tumors undergoing NSAID-induced regression suggests a cause-effect relationship, but it remains to be proven whether or not this represents a primary effect of the drug.

It is possible that by decreasing β-catenin, the tumorigenic defect initiated with loss of full length APC protein could be at least partially corrected and thereby reestablish more normal cellular behavior. Cell migration is impaired by stabilization of the E-cadherin adhesion complex and up-regulation of E-cadherin expression, both of which may occur with increases in cytoplasmic β-catenin secondary to loss of APC (8, 33, 34). It was recently reported that non-neoplastic intestinal epithelium of the Min mouse contains more β-catenin than wild-type mice and that sulindac reduces these levels while increasing the relatively slow rate of cell migration out of the crypt and onto the villus (35). If sulindac modulates β-catenin levels in tumor cells that lack full length APC, as it does in normal Min intestinal epithelium, β-catenin degradation would have to involve processes other than those mediated

by APC and GSK3β (5). It remains to be determined whether this putative NSAID effect is transcriptional in nature, or involves some other constituent of the normal post-transcriptional degradation pathway that might compensate for the lack of APC. Conductin was recently identified as a novel component of the cytoplasmic APC-GSK3β complex and an important contributor in β-catenin degradation (36). Behrens, et al. also showed that over-expression of conductin in SW480 cells decreased levels of cytoplasmic and nuclear β-catenin even though these cells lack full-length APC protein (36). Could NSAIDs up-regulate conductin expression in Min mouse tumors and thereby decreasing β-catenin levels? Alternatively, another recent report showed that proteolysis of β-catenin may occur during apoptosis, apparently due to the activity of a caspase(s) and presumably without the intervention of APC (37).

NSAIDs are currently believed to induce apoptosis in intestinal tumors (12-17). One of the more important apoptosis regulatory molecules is Bcl-2, which inhibits this form of cell death under most conditions (38). Increased levels of COX-2 can result in higher levels of Bcl-2 and resistance of intestinal epithelial cells to apoptosis (39). Since COX-2 may be elevated in tumors of Min mice and FAP patients (14, 30), Bcl-2 could also be contributing to the biology of these lesions.

We found weak staining for Bcl-2 in the normal crypt epithelial cells of the small intestine and stronger reactivity in normal colonic crypt epithelium. Although others have also described Bcl-2 expression in both small and large intestinal crypts (18, 40), some suggest that Bcl-2 is only found in the colonic crypt epithelium (41, 42). While this discrepancy might be explained by variation between individual laboratories in application of

immunohistochemistry, our findings do indicate higher Bcl-2 levels in crypt epithelium of the colon than the small intestine. It is unclear how this distinction might be related to the relative number of lesions that spontaneously develop in the large and small intestines of these mice.

In aggregate, Bcl-2 staining in small intestinal tumors from untreated Min mice was interpreted to indicate over-expression of this protein because of frequent staining (≥ crypt epithelium) in upper regions of the masses, whereas adjacent villus (mature, non-neoplastic) epithelial cells were negative. Colonic tumor cells also stained for Bcl-2 with an intensity comparable to the normal colonic crypt epithelium. As with relative staining intensities in the large and small intestinal mucosa, colonic tumors were more strongly positive than those in the small intestine. One potential implication of this may be reflected in the apparent insensitivity of Min mouse colonic tumors to NSAID-induced regression (22, 24), providing it occurs through apoptosis. There was no indication that Bcl-2 staining changed in colonic tumors after treatment with sulindac. It is not yet clear whether this sustained elevation of Bcl-2 represents a barrier to drug-induced apoptosis, or whether the upstream mechanisms responsible for initiation of apoptosis and down-regulation of Bcl-2 are lacking in these cells.

In contrast to colonic lesions, which contain higher levels of Bcl-2, small intestinal adenomas showed a significant sequential decrease in Bcl-2 staining after 2 and 4 days of sulindac treatment. There was no difference in Bcl-2 scores between untreated tumors and those that had been exposed to sulindac for 20 days. We hypothesize that this later group represents a subset of resistant small intestinal lesions that escape the affects of sulindac (24). When taken together, the colonic and small intestinal data suggest that inherent (or pre-

treatment) levels of Bcl-2 may explain the difference in sensitivity of these two anatomically distinct sets of lesions to sulindac-induced regression. Whether the sustained elevation of Bcl-2 in small intestinal tumors after 20 days of treatment is responsible for their resistance to sulindac remains to be determined. A reduction in Bcl-2 during sulindac-induced regression would, however, be permissive for increased rates of apoptosis (39). In support of a potential relationship between Bcl-2 and sulindac-induced down-regulation of β-catenin, Brancolini, et al. showed that Bcl-2 blocked the proteolysis of β-catenin that accompanied apoptosis (37).

The molecular mechanisms responsible for NSAID-induced regression of intestinal tumors are only starting to be elucidated. *APC* mutation is a key element in the early steps of intestinal tumorigenesis and regulation of β-catenin is an important function of the normal APC protein (5). Our results suggest that the NSAID sulindac may at least partially correct the imbalance in intracellular β-catenin that results from mutational loss of full-length APC protein. If proved true, this suggests a potentially important endpoint in further defining the mechanism of action for sulindac, and perhaps other NSAIDs, in controlling intestinal tumorigenesis. Neither β-catenin nor Bcl-2 levels seem to change in sulindac resistant tumors, but the interaction of these proteins during sulindac-induced regression and any possible link to the arachidonic acid cascade through COX-2 remains to be defined. While it has been suggested that inhibition of prostaglandin synthesis does not fully explain the anti-tumor effects of NSAIDs (15, 17, 24, 32), COX-2 activity clearly has a role in tumor growth and NSAID-induced regression (14, 29). It was recently shown that $PGE_2$ can increase Bcl-2

and inhibit apoptosis in human colonic cell lines (43), but this is discordant with other studies which demonstrate NSAID-induced tumor regression and apoptosis to be independent of eicosanoid levels (15, 17, 24, 32).

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## APPENDIX V

## STRUCTURES AND DOSES OF NSAIDs

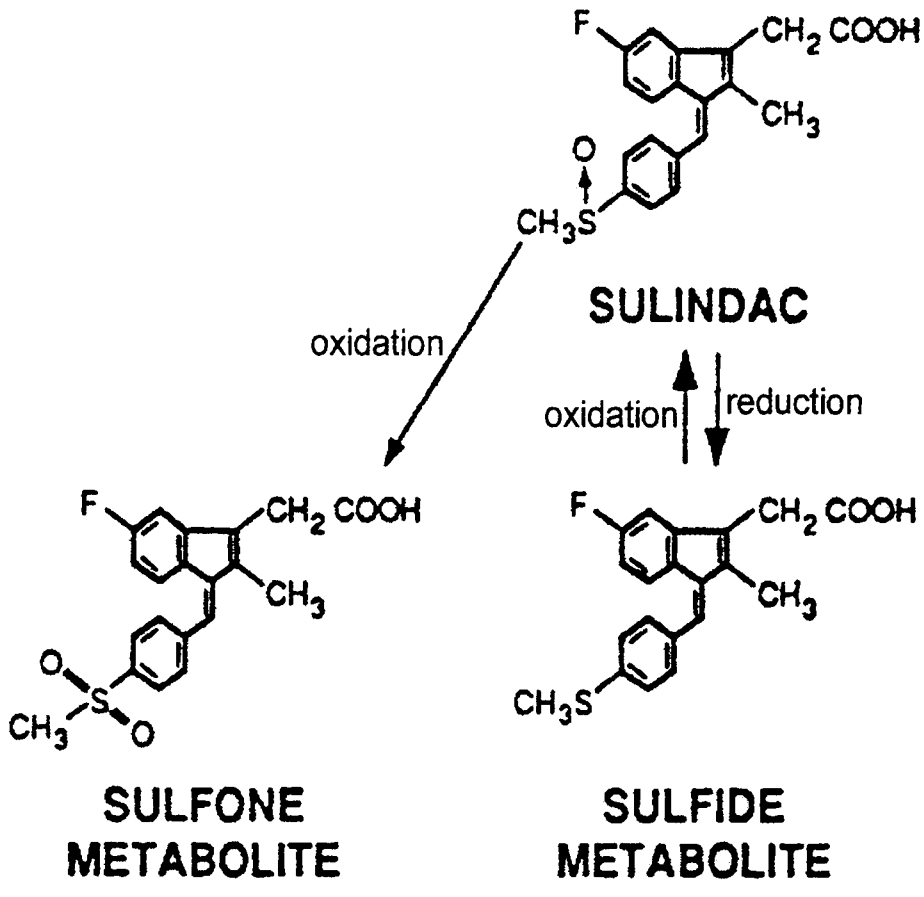
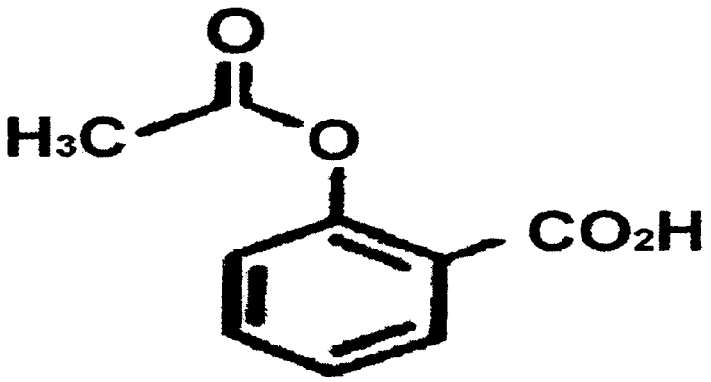
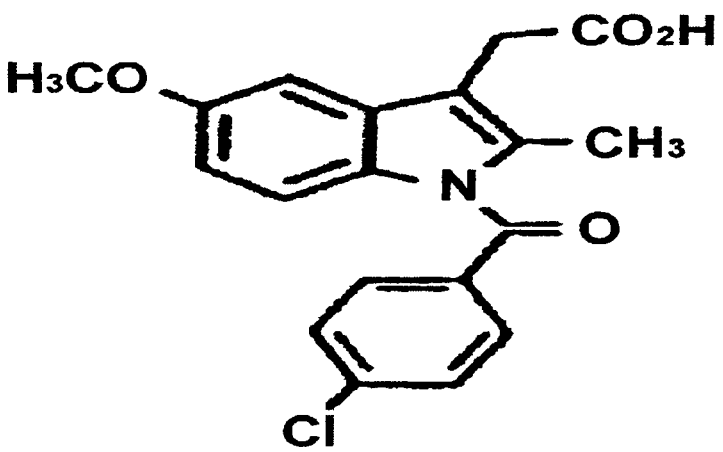



Figure A.7. Chemical structures of sulindac and its metabolites.




ASPIRIN




INDOMETHACIN

Figure A.8. Chemical structures of aspirin and indomethacin.

Table A.2. Doses of sulindac, aspirin and indomethacin

| | Sulindac | Aspirin | Indomethacin |
|---|---|---|---|
| | $C_{20}H_{17}FO_3S$ | $C_9H_8O_4$ | $C_{19}H_{16}ClNO_4$ |
| F.W. | 356.4 | 180.2 | 357.8 |
| ppm | 320 | 400 | 9 |
| mg/mouse/day | 0.83 | 1.06 | 0.023 |
| mg/Kg B.W./day | 30.5 | 43.8 | 0.89 |

# VITA

Chun-Hung Chiu was born to Mr. Ching-Chi Chiu ( 邱靖吉 ) and Mrs. Chun-Wei Shih Chiu (邱施純娓) on August 1, 1962 in Chia Yi, Taiwan, Republic of China (R.O.C.). He attended the elementary school in Min Hsiung, Chia Yi and junior high school in Chia Yi. He graduated from Chia Yi high school in 1980.

He received a Bachelor of Science degree with a major in nutrition from Chun San Dental and Medical College in Tai Chung, Taiwan, R.O.C. in 1986 and served in the military army from 1986 to 1988.

He received a Master of Science degree at North Carolina A&T State University in 1992 and entered the Ph.D. program at the University of Tennessee at Knoxville (UTK) at that time. In July 10, 1993, he married Mei-Shin Mong and they now have a son, Linus Guan-Hu, who was born on June 6, 1998. During his study at the University of Tennessee, he received several honors and awards including the Southeastern Regional Lipid Conference Founder Award in 1996 and Outstanding Research of Graduate Student in Nutritional Science at the University of Tennessee in 1998.

He earned the Doctor of Philosophy degree in August of 1998. He intends to work as a Post-Doctoral fellow in Dr. Jay Whelan's laboratory, in the Department of Nutrition at the University of Tennessee.