ABSTRACT

Title of dissertation: EFFECT OF AGING ON COLONIC CHEMOPREVENTION BY DIETARY CURCUMIN

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The incidence of cancer is highly dependent on age. The hypothesis of this thesis was that aging may alter the efficacy of dietary chemoprevention. This hypothesis was tested by evaluating the effect of age on inhibition of colonic aberrant crypt foci (ACF) by dietary curcumin. Three different ages of male F344 rats were fed either the control diet or diet containing 0.6% curcumin and given injections of a colon carcinogen, azoxymethane (AOM). Curcumin reduced the number of colonic ACF in young and old, but not middle-aged rats. Resistance of middle-aged rats to colonic chemoprevention by curcumin seems to be due to age-related differences in colon carcinogensis rather than curcumin metabolism. Liver cyclooxygenase-2

mRNA expression, measured as an indicator of biological activity of curcumin, was similarly affected by curcumin regardless of ages. Also, curcumin similarly affected arachidonic acid metabolism, which is regarded as one of chemopreventive mechanisms of curcumin, in the colon of young and middle-aged rats.

The involvement of apoptosis was investigated as a potential mechanism responsible for age-related differences in curcumin chemoprevention. A time course study of colonic apoptosis following AOM injections demonstrated that older animals were more susceptible to AOM-induced apoptosis. The effect of aging on curcumin-induced apoptosis in the colon was evaluated at 0, 8, and 16 hours after AOM injection. Curcumin increased both basal and AOM-induced apoptosis in young and old but not in middle-aged rats. Activation of caspase-9 only in young rats fed curcumin indicates that curcumin-induced apoptotic pathway is mediated by mitochondria in young but not in old. AOM may also induce apoptosis by a mitochondrial-independent pathway.

In conclusion, these studies support the hypothesis that aging modulates colonic chemoprevention by curcumin. This dissertation represents the first documentation of an age-related difference in efficacy of dietary chemoprevention. The differential response to curcumin-induced apoptosis is proposed as a mechanism. Further study is needed to confirm whether this phenomenon occurs in humans and

contributes to the lack of agreement between efficacy of dietary chemoprevention in preclinical studies with young animals and clinical studies with adult humans.

EFFECT OF AGING ON COLONIC CHEMOPREVENTION BY DIETARY CURCUMIN

By

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Dissertation submitted to the Faculty of the Graduate School of the University of Maryland, College Park, in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
2006

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Acknowledgements

My thesis could not have been possible without many people that have been accompanied and supported. It is a pleasure that I have an opportunity to express my gratitude for all of them.

First of all, I would like to thank my advisor, Dr. Bernadene Magnuson. During my Ph.D. study, I even asked her why she put me in this challenging project. She guided and encouraged my study with great enthusiasm and patience. Besides being an excellent research advisor, she was always there for listening my frustrations and providing helpful comments on my future career. I thank that we shared lots of memories not only in the lab but also out of campus. I am really glad that I was guided by Dr. Magnuson and have become to know her.

I am grateful for current and previous members of Dr. Magnuson's lab. It was a pleasure to work with them. A special thanks goes to Dr. Minnie Malik who helped me to learn molecular techniques. I learned from her how to do Western Blot even after she moved to Uniformed Services University. I also thank James Montgomery and Lee Unangst for their help in animal studies.

I would like to thank Dr. Sabine Francke in FDA for her pathology work. It was always nice to have her comments on morphological and pathological

observations and work with her nice microscopes. I also thank Dr. Laura Kresty in Ohio State University who offered me to image my slides and analyze cell proliferation.

Besides my advisor, I would like to thank the other members of my thesis committee, Dr. Harry Swartz, Dr. Thomas Castonguay, Dr. Mark Kantor, and Dr. Thomas Wang for providing me provocative and valuable comments. I luckily had a nice and supportive committee and have learned from them what is being a scientist. Especially, I thank Dr. Castonguay who has a great enthusiasm in giving encouragement and learning to students. I am glad that I was one of them.

Let me also say “thank you” to my friends who were in our department during my study, especially, Ye Su, Emily Ye, Yifan Zhang, Pu Jing and Zian He. It was always joyful to get together with them.

Lastly, but not least, I thank my family in Korea. I appreciate their lots of prayers for me and the way they love me. I was sorry that I was not with them to share moments of their happiness and sadness. As my Mom told me, I am not alone and they are always with me. That is why I could be stronger than I am. I thank that I have wonderful siblings, Sanghui, Eunjung, and Woosung. I thank Sanghui who already went through Ph.D. degree in Nutrition for her helpful advice on achieving a degree. I hope that we can conduct interesting works together in the

future. I also thank my brave sister, Enujung who was on my side when I decided to come to the US for my Ph.D. study. I cannot forget my little brother, Woosung. Being aware of being his big sister, I could overcome many moments which tempted me to give up.

One says that Ph.D. is not a work of one person. I am greatly thankful that I was lucky enough to have these people and accomplish this journey. Once again, I thank all for their help and support and making possible this journey.

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Chapter 1. Literature Review

1.1 Cancer: a disease of the elderly

Cancer is often characterized as a multi-step process, including initiation, promotion and progression (1). Exposure to carcinogens induces mutations either directly or through formation of DNA adducts. DNA adducts can cause mutations and mutations left without repair can be permanently fixed by DNA replication. If this mutation occurs at genes that regulate cell growth and sites critical for protein function, cancer can be initiated. However, cancer initiation is not enough to develop tumors. Promotion to tumors involves selective growth of initiated cancer cells. This step is mediated by promoting factors such as inflammatory responses and hormones. As the tumor grows, cancer cells acquire more mutations, lose cellular differentiation, and tend to metastasize. The acquisition of the ability to metastasize results in progression to metastatic tumors.

The incidence of cancer is highly dependent on age (2). In humans, cancer incidence exponentially increases with age from age 40 to 80 (3). More notably, the majority of cancers in the older ages are derived from epithelial cells (3). Although it is unclear how the aging process is involved in carcinogenesis, there are several suggested mechanisms to explain the marked rise of cancer incidence in the aged.

Sequential accumulation of somatic mutations over a lifetime has long been considered to contribute age dependence in cancer incidence. This view has recently been reinforced in the reviews by Peto (4) and Dix (5). According to this hypothesis, we are continuously exposed to various endogenous and exogenous DNA damaging agents through the lifetime. The high prevalence of cancer in the older subjects simply reflects a more prolonged exposure to carcinogens. Cancer is a disease of genes as described above. Also, somatic mutation due to oxidative damage, an endogenous carcinogen, increases with age (6). However, frequent mutations are also observed in normal appearing tissues (2). Moreover, this hypothesis may not be enough to explain why epithelial cell driven cancers are predominant in older individuals (3). Therefore, it is unlikely that overload of somatic mutation alone in the aged is sufficient to drive cancer.

There is increasing evidence that the cellular microenvironment is a potent factor to determine suppression or promotion of malignant phenotype (2, 3, 7). Successive studies of McCullough et al. (8-10) showed that the development of tumors from epithelial cancer cells was affected by changes in tissue microenvironment and that the aging process causes such alterations in the tissue environment. In their studies, intrahepatic transplantation of liver neoplastic epithelial cells rapidly produces tumors at the sites of inoculation both in young and

old rats. However, tumors at the transplantation site in young rats eventually regress, whereas in older rats, tumors grow progressively (8). McCullough et al. also found that liver neoplastic epithelial cells transplanted in extrahepatic sites did not regress, even in young animals (9). Cells transplanted to the liver reside as differentiated hepatocytes. Upon withdrawal from the liver, neoplastic epithelial cells revert to an undifferentiated and malignant phenotype (10), indicating the importance of environmental signals to suppress malignancy. Krtolica et al. (11) also reported that senescent cells stimulate growth of premalignant cells but not normal cells in culture. Senescent fibroblasts induce premaligant and malignant cells to form tumors *in vivo* (11). These findings suggest that the microenvironment can be a powerful modulator of tumor development from initiated cancer cells. More importantly, the aging process may modulate tissue environment, promoting cancer cells to develop malignant tumors. Thus, the age-related increase in cancer results from the interplay of both accumulation of mutations and alteration of tissue milieu to promote tumor development with age, as suggested by Krtolica et al. (2, 11).

1.2 Diet and colon cancer

Colorectal cancer is a leading neoplastic disease that affects both men and women in high frequency in Western countries, including the United States (4).

Cancer statistics for 2005 demonstrate that colon cancer is the third most common cancer both in men and women (12). About 104,950 new cases of colon cancer are expected to be diagnosed and about 56,290 men and women together are expected to die of cancer of colon and rectum in 2005 (12).

Cancer risk is associated with both inherent factors such as mutations and environmental factors including diet, tobacco use, and physical activity (4). Interestingly, colon cancer risk is closely related with dietary factors. For example, red meat consumption appears to increase the risk of colorectal cancer (13). There is converging agreement that heme may be responsible for the increase of colon cancer by high consumption of red meat (14, 15). Several case-control studies (16, 17) showed that colorectal cancer risk was positively correlated with high consumption of well-done meat probably due to heterocyclic amines (HCA) produced by pyrrolysis of meats when cooked at high temperature (18). In addition, this relationship was further enhanced when genetic polymorphism of HCA-metabolizing enzymes was considered (16). Dietary fat has been considered to increase cancers related with Western lifestyle, including colorectal cancer (19). However, not only amount of consumption but also the fatty acid composition appears to contribute to the increase of colon cancer (20). Generally, n-3 polyunsaturated fatty acids are thought to protect against colorectal cancer (21, 22) whereas n-6 polyunsaturated fatty acids

appear to promote colon carcinogenesis (21, 23).

On the other hand, some nutrients and phytochemicals show a preventive effect on colon cancer. Epidemiological studies showed that high consumption of fruits and vegetables is negatively associated with colon cancer risk. As a result, plant foods and compounds derived from plants have been widely investigated in relation with prevention of colon cancer (24-27). Several prospective studies showed that high calcium consumption has a negative relationship with risk of colon cancer (28, 29). Sesink et al. (14) suggested that dietary calcium inhibits colon carcinogenesis by inhibiting the hyperproliferative effect of dietary heme. It is generally considered that dietary intake of folate is inversely related with colorectal cancer risk due to its role in one carbon transfer in DNA synthesis (30, 31). Polymorphism in the methylenetetrahdrofolate reductase gene, which causes DNA hypomethylation, seems to modulate colorectal cancer risk when folate status is low (30, 31), showing a diet-gene interaction in colon carcinogenesis. A diet-diet interaction in colon carcinogenesis also has been reported by Lupton and colleagues (32). Their study suggested that fish oil may be protective against experimentally-induced colon carcinogenesis by enhancing apoptosis when compared to corn oil diets, but this depended on the dietary fiber included in the diet (33). Therefore, as we further understand diet-gene interactions as well as diet-diet interactions, it is

becoming more apparent that dietary components greatly impact colon carcinogenesis.

1.3 Colon cancer prevention study in animal models

Reliable preclinical models are critical in understanding etiology, development, and control of human diseases including colorectal cancer (34). In fact, animal model studies of colon cancer have contributed considerably to our understanding of colon carcinogensis and evaluation of nutritional and chemopreventive agents for the prevention of colon cancer. The $APC^{min/+}$ mouse model and the azoxymethane (AOM) rat models are the main animal models used to study development and prevention of colon cancer (34).

Reddy and colleagues (34) have largely contributed to establishing the AOM rat model. Using this model, they have tested many potential chemopreventive agents. AOM is an alkylating agent. After AOM is administered, methyl groups of AOM are hydroxylated to azoxymethanol (MAM), probably by CYP2E1 and other cytochrome P450 enzymes (35). MAM can be further oxidized in the liver and extrahepatic organs, including the colon, producing methylazoxyformaldehyde that produces the highly electrophilic methyldiazonium ion. Methyldiazonium ions eventually methylate DNA and form DNA adducts prone to mutation (35). Other alkylating agents including 1,2-dimethylhydrazine, a precursor of AOM, also have

been used to induce colon cancer in specific strains of rats (36-39). Direct acting carcinogens, such as methylnitrosourea or N-methyl-N'-nitro-N-nitrosoguanidine are used for chemically-induced animal models (40, 41). Heterocyclic amines, like 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), is another class of chemicals that has been used as a colon cancer inducing agent. In contrast to AOM, PhIP is present in our daily foods (42, 43). It has been argued that PhIP-induced colon tumor model is a more appropriate model for mimicking human colon cancer studies (43). However, AOM has been extensively used by many investigators as it is less expensive, more potent and more convenient to use (34).

If AOM is given weekly twice subcutaneous (s.c.) injection at the dose of 15 mg/kg body weight, the first visible colon tumors can be endoscopically detected 15 weeks after the treatment and the mean latency period of such tumors is about 20 weeks (44). It has been demonstrated that AOM-induced tumors share many morphologic and histopathologic characteristics with human tumors (34). In addition, the biological behavior of AOM-induced rat colon carcinomas is similar to that of human colon carcinomas (34). AOM-induced carcinomas metastasize to regional lymph nodes and the liver. Also, as in human colon cancer, both adenomas and adenocarcinomas occur in the AOM-rat model (34). Genetic mutational changes in AOM-induced colon have been largely studied by Takahashi and colleagues (45-

48). In AOM-induced colon tumors, mutations in *K-Ras* gene are as frequent as in human colorectal tumors (45, 48). However, in contrast to human tumors, *Apc* gene mutations are rarely observed in AOM-induced tumors (49). *Apc* mutations have been found in patients with familial adenomatous polyposis (FAP) who have germline mutations in one of the *Apc* alleles as well as in sporadic colon cancer (50, 51). The protein products of mutant *Apc* genes are defective in their ability to stimulate degradation, resulting in stabilization and accumulation of β-catenin in plasma (52). Interestingly, Takahashi et al. (46, 47, 53) reported that *β-catenin* genes are frequently mutated in AOM-induced colon tumors, suggesting that similar pathways are involved in both human and AOM-induced rat colon carcinogenesis.

The multiple intestinal neoplasia (Min) mouse was first identified by Moser et al. (54). This Min mouse has mutated *Apc* genes in heterozygous forms and mimics the rapid development of adenomatous polyps as shown in humans with FAP (55). After the discovery of the Min mouse with truncated *Apc* in position 850, other genetically modified models have been developed such as mice with mutations in different location of the *Apc* gene or other cancer related genes like *Msh2* and *Mlh1* (50). This Min mouse model develops a large number of tumors within short time. Min mice on a C57BL/6J background develop an average of more than 50 tumors within 90 days of birth (55). However, the major drawback of this mutant model is

that tumors occur predominantly in the small intestine, not in the colon (50). Moreover, most tumors in the Min mouse are adenomatous whereas adenocarcinomas, more invasive tumors, are seldom observed (34). In addition, the *K-Ras* mutations observed in many human tumors were not detected in Min mice tumors (50). In spite of these drawbacks of the Min mouse as a model of human colon cancer, this model has provided a shorter assay time to evaluate tumors and has made a contribution to the understanding of the colon cancer process and prevention by diets and chemopreventive agents.

Corpet and Pierre (50) recently reported in their review that there is a correlation, in general, between the efficacy of diets or chemopreventive agents in the Min mouse model and the AOM rat model. They concluded that there is a close agreement between the results observed in the colons of the AOM rat model and in the small intestine of the Min mouse model. They also examined the relationship of animal studies with human trials. When they compared the results of colon tumor incidence in AOM-treated rats or polyp number in Min mice to the recurrence of colonic adenomatous polyps in human trials, many promising chemopreventive agents which strongly and consistently suppressed colon carcinogenesis in animal models showed at best modest effect in inhibiting colon carcinogenesis in humans. There are differences in end points, cancer incidence *vs.* recurrence of polyps, and other

limiting factors in human studies such as compliance to the chemopreventive agents and errors in sample selection as addressed by Corpet and Pierre (50). Also, differences of pharmacokinetics of chemopreventive agents caused by species difference may contribute to the efficacy of agents in inhibiting colon cancer. However, one of main differences that have not been considered may be the age of the target group. Human intervention trials with chemopreventive agents target middle to older adults, whereas preclinical studies have been exclusively conducted with young animals. As described earlier, aging is perhaps the most potent carcinogen which drives cancer including colorectal cancer, and tissue milieu altered with age appears to promote tumor development. Consequently, the effect of age on chemoprevention should be considered in interpretation of preclinical results to human trials.

1.4 Aberrant crypt foci as a biomarker of colon cancer

To investigate the effects of dietary factors on colon cancer development in animal models, evaluation of the number, size, and the extent of aggressiveness of tumors are used. Min mice, which rapidly develop tumors, rarely live more than 150 days of age on the c57BL/6J background due to pathologies other than colon cancer including anemia and intestinal blockage (54). As a result, this model cannot be

used in aging studies. However, a long period of time is required to develop colon tumors when tumors are induced with chemical agents as in the AOM rat model. Consequently, surrogate end point biomarkers have been sought to predict tumor incidence. Aberrant crypt foci (ACF) have been regarded as precancerous lesions and have been widely used as a biomarker of colon cancer in many studies (56).

Bird (57) first identified ACF from the colon mucosa of mice treated with AOM and defined them as crypts that have altered luminal opening; exhibit thickened epithelia; and are larger than adjacent normal crypts. ACF are observed in rodents administered colon chemical carcinogens but are not present in the colon of untreated animals (56). However, there is a report (58) that ACF were observed in old untreated rats, but not in young. Later, Pretlow et al. (59) also found the presence of such lesions in the colon of patients with colon cancer. Identification of ACF lead to a number of studies to evaluate molecular, morphological, and growth features of ACF and to assess their nature as precursor lesions of colon cancer (56). The number and growth of ACF are enhanced or suppressed in response to known promoters or inhibitors of colon cancer (60, 61). It has been reported that ACF harbor mutations in *K-Ras* genes both in rodent and human colons as often observed in colon tumors (46). Increases of inducible nitric oxide synthase have also been observed in AOM-induced ACF and in human colon tumors (47). Hyperplastic ACF,

which are less advanced, have a higher tendency to harbor *K-Ras* mutations and no *β-catenin* mutations whereas dysplastic ACF, which are more advanced, showed altered cellular localization of β-catenin (46). Hao et al. (21) also reported altered β-catenin expression in human colonic aberrant crypt foci.

Important findings in interpreting ACF data were made by a limited sequential analysis of the number and growth of ACF prior to and at the time of tumor appearance. Magnuson et al. (62) showed that total number of ACF was decreased not increased in rat colons by feeding of diet containing 0.2% cholic acid, a known colon cancer promoter. However, when the number of ACF were examined separately by crypt multiplicity (number of crypt per focus), ACF with large multiplicity were increased by cholic acid diet over time whereas ACF with small multiplicity decreased as duration of ACF increased, suggesting selective stimulation of ACF by cholic acid. Recently, Papanilolaou et al. (63) also suggested that large ACF are more relevant to predict tumor outcome. They sequentially analyzed ACF and tumors in three strains of mice differing in susceptibility to AOM in developing colon cancer. In their study, almost no differences were found among different strains of mice in total number of ACF whereas large ACF (5 and over crypts/focus) were correlated with tumor incidence and susceptibility to AOM-induced colon tumors. Moreover, morphological analysis of ACF showed that most susceptible

mice had the highest percentage of dysplastic ACF (the more advanced forms), followed by the relatively susceptible strain, indicating that the difference in susceptibility may be due to the lack of progression of smaller ACF in the resistant mice (63).

There is a general agreement that ACF are preneoplastic lesions of colon cancer. The model of genetic changes associated with human colorectal tumorigenesis (64, 65) supports hypothesis that ACF are precursor lesions and that carcinomas arise from preexisting dysplastic ACF. In this model, *Apc* mutations initiate the neoplastic process and develop into numerous dysplastic ACF. Some, but not all, ACF progress to adenoma as they acquire *K-Ras* mutations and develop to carcinoma as they acquire additional mutations including mutations in *p53* (65, 66). However, it should be noted that the majority of ACF will not develop into colon tumors and that ACF are heterogeneous at the morphological and molecular levels. Therefore, ACF with large multiplicity or advanced ACF should be considered predictive of tumor incidence when ACF are utilized as a biomarker of colon cancer although evaluation of tumors is the best way to investigate the effect of dietary factors on development of colon cancer.

1.5 Curcumin and colon cancer prevention

Many compounds of plant origin have been studied in relation to their biological activity. Curcumin is one of the compounds that have been extensively studied in a broad spectrum of biological activity including antioxidative activity, lipid and cholesterol lowering activity and anti-inflammatory activity (67-69). Many studies also showed potential chemopreventive activity of curcumin in many different types of cancer such as skin, colon, breast, prostate, lung and liver (70-75). Some studies also indicated potential combinational use of curcumin to improve efficacy of other cancer treatments as shown in adjunct use with TRAIL-mediated immunotherapy (76), and to reduce treatment-induced toxicity as observed in radioprotective effect of curcumin when curcumin is given before or after γ-ray irradiation (77).

Curcumin is a polyphenolic compound imparting the yellow color in the spice turmeric (78). Turmeric, powdered rhizome of *Curcuma longa* Linn., has been extensively used for flavor and color in food preparation and also for treatment of inflammatory conditions and other diseases in East Asia (78). In India, one of the countries that consume high amount of curcumin in their diet, the rates of colorectal, prostate, and lung cancer are among the lowest in the world (79). During its long history of usage in diet, almost no toxicity of curcumin has been reported. A recent

pharmacological study of curcumin also showed that there is no dose-limiting toxicity at doses between 450 and 3,600 mg per day for four months in colon cancer patients (80). At least two phase I curcumin clinical trials were completed or are ongoing (80, 81) and curcumin is in a phase II clinical trial for the prevention of colorectal cancer.

Many studies using human cell lines and animal models have demonstrated chemopreventive activity of curcumin against colon cancer. Feeding 0.2% curcumin to F344 rats inhibited both AOM-induced ACF formation (82) and tumor development (71, 72). Curcumin also decreased incidence of intestinal tumors in the Min mouse model (27, 42). Inhibition of cell growth by curcumin was observed in different colon cancer cell lines with different genotypes and phenotypes including HT-29, HCT116, SW620, and SW480 (83-85). These studies with preclinical models also suggested potential chemopreventive mechanisms through which curcumin acts.

Earlier studies investigated anti-inflammatory activity of curcumin in relation with mechanisms of colon cancer prevention. Cyclooxygenases (COX) are enzymes that mediate inflammatory process, catalyzing conversion of arachidonic acids into prostaglandins (86). Two isoforms of COX are known, COX-1 and COX-2. COX-1 is constitutively expressed, maintaining normal physiological function. In contrast, COX-2 is induced by cytokines, mitogens and tumor promoters. Many studies have

reported that COX-2 is highly expressed in colon tumors (87-89) and inhibition of COX-2 is related with colon cancer prevention (90). Curcumin inhibited phospholipases, responsible for generation of arachidonic acid and formation of prostaglandin E2 both in colonic mucosa and colon tumors (71). Goel (91) also reported that curcumin inhibited COX-2 expression at mRNA and protein levels but did not affect COX-1 expression in HT29 cells.

Curcumin is also known to inhibit activation of nuclear transcription factor κB (NF-κB) by inhibiting I-κB kinase (IKK) activity (83). NF-kB is present in the cytoplasm as an inactive form bound with its endogenous inhibitor, IκBα. Following stimulation by cytokines, IκBα is phosphorylated by successive kinases including NF-κB inducing kinase (NIK) and IKK and undergoes rapid degradation, resulting in activation of NF-κB and triggering gene transcription related with cell survival (92, 93). In addition, studies with cell lines derived from colon cancer have showed that curcumin modulates cell signaling pathways, inhibiting cell proliferation as well as inducing apoptosis (92, 94-96).

1.6 Induction of apoptosis: one of chemopreventive mechanisms of curcumin

Apoptosis, programmed cell death, is the process by which cellular proteins and DNA decompose in an orderly manner. Cells are subsequently taken up by

phagocytosis. In mitotic tissues such as the colon where cells continuously proliferate, cell number is maintained by apoptosis in coordination with cell proliferation (97). Apoptosis also plays an important role in removing unwanted cells caused by severe DNA damage which otherwise may develop into a neoplasm, thereby preventing carcinogenesis (98, 99). Indeed, apoptosis is one of main mechanisms through which many chemopreventive agents, including curcumin, act.

Apoptosis molecular pathways were described well in a recent review by Sprick and Walczak (100). Mainly, two different pathways leading to apoptosis have been identified – intrinsic and extrinsic pathways. In spite of their different modes of apoptosis induction, both pathways involve the activation of a cascade of proteolytic enzymes known as caspases. In the extrinsic pathway, also called death receptor-mediated pathway, cell death is initiated by stimulation of members of the tumor necrosis factor (TNF) receptor superfamily, termed death receptors, in plasma membranes. Binding of death receptors by their ligands triggers intracellular binding of the adaptor-protein Fas-associated death domain (FADD) to the receptor, forming the death inducing signaling complex (DISC). FADD serves to recruit pro-caspase-8 into the complex, resulting in activation of pro-caspase-8. Caspase-8 triggers the activation of caspase-3. In contrast, the intrinsic pathway is mediated by the mitochondria that regulate the formation of the caspase-9-activating complex, the

apoptosome. Chemical stress is one of stimulants to trigger the formation of apoptosome by causing a breach in mitochondrial integrity and therefore a release of cytochrome *c* and other pro-apoptotic molecules. Released cytochrome *c*, together with Apaf-1, recruits pro-caspase-9 to activate it and later trigger the activation of caspase-3.

Many proteins are involved in the regulation of the apoptosis pathway. The integrity of the mitochondrial membrane is regulated by the Bcl-2 family, which can be divided into three groups, anti-apoptotic, pro-apoptotic, and BH-3-only proteins (100). Anti-apoptotic Bcl-2 members, like Bcl-2 and Bcl-x, serve to maintain membrane integrity. Under normal conditions, BH-3-only proteins are inactive. Upon activation, they activate pro-apoptotic proteins, Bax and Bak,that breach mitochondrial integrity. Pro-apoptotic Bid that belongs to BH-3-only proteins is activated by caspase-8-mediated cleavage, providing a link between the receptor-mediated pathway and mitochondria-mediated pathway (100). Also, apoptosis can be negatively regulated by cellular caspase-inhibitors such as IAPs that inhibit activation of caspases (100). Heat shock proteins induced by various stresses are known to play a negative regulatory role in apoptosis (101, 102). In addition, apoptosis is mediated or suppressed by other cell signaling pathways including c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), and NF-κB

pathways (84, 92, 103). Therefore, cumulatively cell destination, death or survival, is determined.

Many studies with different types of cells have showed that curcumin induces apoptosis and inhibits cell growth. Curcumin, at a concentration of 10 μM, enhanced apoptosis in the breast cancer cell line, MCF-7, by p53-dependent Bax induction (74). In human melanoma cells, apoptosis was induced by 60 μM curcumin through Fas receptor pathway, p53-independent (104). On the other hand, 25 μM curcumin induced apoptosis through activation of BID cleavage and cytochrome *c* release in the myelogenous leukemia cell line HL-60 (96). Treatment of renal carcinoma Kaki cells with curcumin downregulated Bcl-2, Bcl-xL, and IAP, activating the release of cytochrome *c*, resulting in induction of apoptosis (105). In HCT116 colon cancer cells, curcumin-induced apoptosis was mediated through JNK, a member of MAPK. Curcumin treatment induced sustained activation of JNK and phosphorylation of c-Jun and a JNK-specific inhibitor prevents curcumin-induced apoptosis (84). Therefore, curcumin-induced apoptosis is mediated by many different signaling pathways and this is largely dependent on the types of cells and concentration of curcumin.

Chapter 2. Effect of age on inhibition of ACF development by curcumin

2.1 Introduction

Colorectal cancer, one of the leading causes of deaths in the United States, is strongly associated with dietary factors. Many compounds in food, including some nutrients, have been reported to promote colon cancer whereas some nutrients and many phytochemicals have been shown to have colon cancer preventive properties (106, 107). Curcumin, the yellow pigment of rhizomes of *Curcuma longa* Linn., has been commonly used for flavoring and coloring as a powder called turmeric in South East Asia. Curcumin has been gaining popularity as a supplement due to reported various biological activities that include its anti-inflammatory, antioxidative, anti-bacterial and chemopreventive activities (78).

Curcumin inhibits colorectal cancer in several animal models. Feeding of 0.2 % curcumin to azoxymethane (AOM)-treated F344 rats inhibited both incidence of colon tumor and multiplicity of tumor (71, 72). Curcumin also decreased intestinal tumors in a familial adenomatous polyposis animal model (27, 42). The study by Sharma et al. (108) provided preliminary information on human pharmacodynamic and pharmacokinetic properties of curcumin extract in patients

with colorectal cancer.

However, preclinical studies aimed at identifying potential chemopreventive dietary compounds have been conducted almost exclusively with young animals. Curcumin is no exception. Modulation of gene expression and oxidative stress through which dietary compounds may show chemoprevention are highly associated with age (109, 110). Moreover, metabolism of curcumin, which can be affected by the age, may alter efficiency of chemoprevention by curcumin (111). For these reasons, the current animal study design using young animals may not properly reflect chemopreventive activity of dietary compounds in human colon cancer, a disease of the aged. The hypothesis of this study was that maturation and aging may alter the response to a dietary intervention. This hypothesis was tested using curcumin, a well-established colon chemopreventive agent, and the aberrant crypt foci (ACF) model. ACF are preneoplastic lesions of colorectal cancer and ACF with large multiplicity are closely associated with tumor development (62). This study was conducted to investigate the effect of age on inhibition of early stage of colon cancer by curcumin.

Cyclooxygenase (COX) enzymes catalyze conversion from arachidonic acids to prostaglandins (86). Curcumin reduced COX activity in colonic mucosa and tumors from AOM-treated F344 rats (71). COX-2 is highly expressed in colon

cancer and prevention of COX-2 expression suppresses progression of colon tumor (91, 112). Zhang et al. (113) reported that curcumin inhibited bile acid and phorbol ester-induced COX-2 expression in gastrointestinal cell line. In addition, curcumin specifically inhibited COX-2 expression in HT-29 cells whereas COX-1, constitutively expressed in most tissues, was not affected by curcumin (91). As a result, colonic COX-2 expression levels were measured using immunohistochemistry in this study.

We also investigated whether the age-related difference in response to dietary curcumin was colon specific. Curcumin affects arachidonic acid metabolism in the liver and inhibits formation of AOM-induced prostaglandins (114). Ramirez-Tortosa et al. (69) reported that an ethanol-aqueous extract obtained from curcumin lowered total plasma cholesterol in male rabbits fed 1.3% cholesterol diet. Administration of 500 mg of curcumin per day for 7 days reduced total serum cholesterol in healthy humans (115). Therefore, liver COX-2 mRNA expression and total serum cholesterol were measured to determine whether age affected other tissues in response to dietary curcumin. This study reports significant effects of age on response to dietary curcumin in F344 male rats.

2.2. Materials and Methods

2.2.1 Experimental animals and diets

Experimental animals were young (6 weeks), middle-aged (12 months), and old (22 months) male F344 rats obtained from the colony at the National Institute of Aging (Bethesda, MD). Six rats of each age group were randomly assigned to either AIN-93 containing 0.6% curcumin (Sigma, St. Louis, MO) or AIN-93 control diet. For the old group, 8 rats were allotted to each diet group because of their high risk of loss. One week after starting on the experimental diet, all the rats were treated with azoxymethane (AOM, Sigma, St. Louis, MO) with 2 weekly s.c. injections (15 mg/kg). Rats were fed experimental diets for total 3 months, then were killed and colon, liver and serum were collected. In the old group fed AIN-93 diet, two months after the first injection of AOM, one rat was found dead and another rat was killed because of significant weight loss. Two of old rats fed curcumin showed lower food consumption and body weights through the whole experiment even though they recovered their body weight within 10 days after AOM injections. Therefore, two old rats in each diet group were excluded from the data analyses.

2.2.2 Aberrant crypt foci evaluation

Whole colons were flushed with 1 X phosphate buffer saline and opened. Colon pieces fixed in 4% paraformaldehyde were stained with 0.2% methylene blue as previously described (116). Number, multiplicity (number of crypts per focus) and distribution of ACF were recorded. ACF with 5 and 2 multiplicity in a colon stained with methylene blue are shown in Fig. 1. ACF were evaluated from whole colon except for a short segment taken from the middle 2 cm toward the cecal end. This segment was used later for RNA isolation. The RNA obtained from that tissue was of poor quality and could not be subsequently used to study changes in gene expression as originally intended.

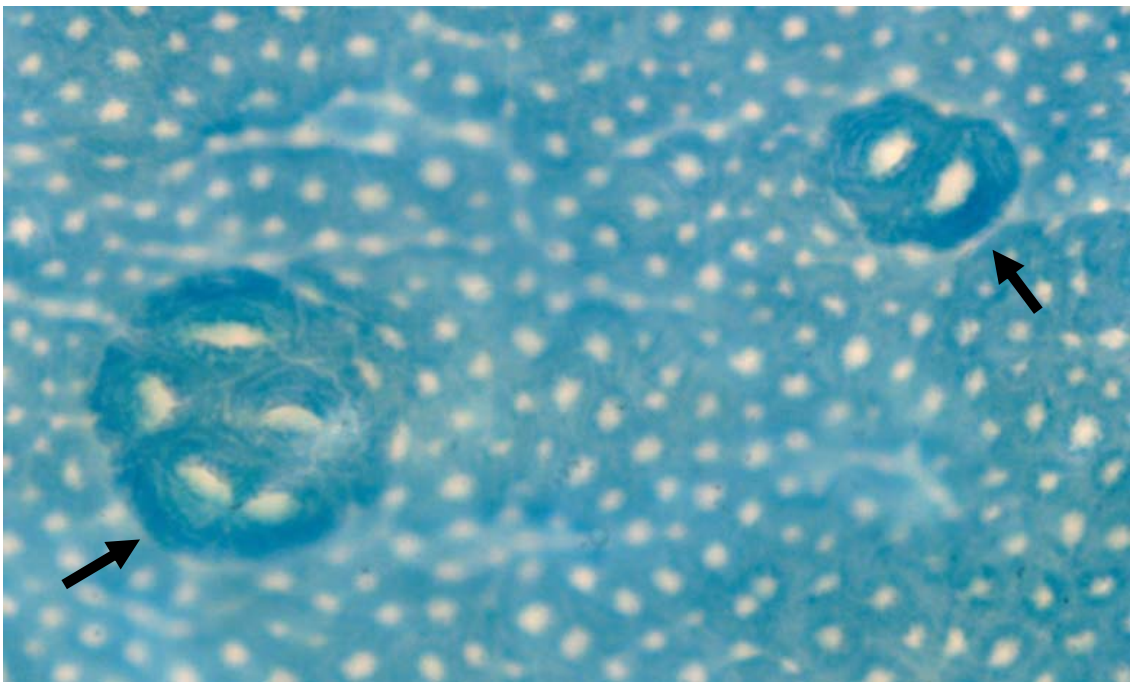

Figure 1. Aberrant crypt foci (arrows) in a rat colon stained with methylen blue. Focus with 5 multiplicity is on the left and 2 multiplicity on right.

2.2.3 Liver cyclooxygenase-2 mRNA expression

Total cellular RNA from liver was extracted by the use of TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA samples were treated with DNAse-I enzyme using the DNA-*free* kit (Ambion Inc., Austin, TX). First strand synthesis was performed using Retroscript kit (Ambion Inc.) and concentration of cDNA was measured spectrophotometrically.

Cyclooxygenase-2 gene expression was studied using mouse COX-2 gene-specific Relative RT-PCR Kit (Ambion Inc.). Ribosomal gene 18S (498 bp) was used as an internal control. The 18S primer:competimer ratio was optimized according to the manufacturer's instructions before changes in expression of COX-2 gene (297 bp) were analyzed. After optimization of PCR assay conditions (DNA Engine, MJ Research, Waltham, MA), the multiplex reaction contained 1X complete reaction buffer; 200 µM dNTPs mixture; 0.4 µM 18S primers:competimers (1:9) mixture; 0.4 µM COX-2 primer and 0.5 U/25 µl *Taq* DNA polymerase. The following thermocycling conditions were used for PCR assays: one 2-min cycle at 92 ^{o}C followed by 27 cycles of denaturation for 30 s at 92 ^{o}C, annealing for 30 s at 59 ^{o}C, and extension for 1 min at 72 ^{o}C. The final extension was given for 5 min at 72 ^{o}C before analysis of the PCR products.

The PCR products from multiplex reactions were quantified using DNA 500

LabChip® and Agilent 2100 bioanalyzer according to the manufacturer's protocol. Changes in the gene expression were determined by calculating the ratio of COX-2 mRNA to 18S mRNA. The ratios were calculated from the area under the curve values for each PCR product.

2.2.4 Cyclooxygenase-2 immunohistochemisty

To determine whether age or diet affects expression of COX-2 gene product in the colon, four pieces of tissues from different locations of the formalin-fixed colons were longitudinally embedded in paraffin, sectioned and immunostained for COX-2. Deparaffinized tissue sections were incubated with 3% hydrogen peroxide and 2% normal goat serum to block endogenous peroxidase activity and non-specific binding sites, followed by incubation with rabbit polyclonal anti-murine COX-2 antibody (Oxford BioMedical Research, Oxford, MI) diluted 1:1000. Biotinylated anti-rabbit igG incubation was followed by amplification using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA). Color was developed by treatment with diaminobenzidine tetrahydrochloride (DAB, Sigma). Slides were counterstained with hematoxylin.

2.2.5 Total serum cholesterol

Blood was collected by cardiac puncture and centrifuged at 3000 rpm for 20 minutes after clotting. Collected serum was frozen at -80 ^{o}C until analysis. Serum cholesterol was measured with the RefLab® cholesterol reagent (Fisher Scientific, Pittsburgh, PA) using a Beckman 640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

2.2.6 Data analysis

One-way analysis of variance (ANOVA) was conducted for the all data analyses using SAS software (8.1, SAS Institute Inc, Cary, NC) to determine if there were significant differences between rats fed curcumin and control diets. Two-way ANOVA was performed to examine the effect of age and diet and their interaction on the number of ACF with 2 and over multiplicity. Repeated measured analysis was used for average body weight at each time point.

2.3 Results

2.3.1 General observations

Average food consumption of each group of rat is shown in Table 1. Curcumin did not change food consumption in either middle-aged or old rats. However, food consumption was slightly higher in young rats fed curcumin ($p < 0.05$) compared to young rats fed the control diet. When repeated measured analysis was performed for each time period, there was no difference ($p > 0.05$) in body weights of rats fed AIN-93 control diet (AIN) and AIN-93 containing 0.6% curcumin diet (CUR) in all age groups (Fig. 2). Both middle-aged and old rats took longer than young rats to regain weight loss resulting from the AOM injections (Fig 2).

Table 1. Food consumption of different age groups of rats

Diet[a)] \ Age	Young	Middle-aged	Old
AIN	13.9 ± 0.3[b)]	14.9 ± 0.5	13.3 ± 0.5
CUR	15.2 ± 0.5[c)]	14.8 ± 1.0	14.3 ± 0.5

[a)] AIN represents the group fed the AIN-93 control diet and CUR represents the group fed the AIN-93 diet containing 0.6% curcumin

[b)] g/day, mean ± SE, n=5-6 rats/group

[c)] significant difference ($p < 0.05$) between diet groups in each age group by ANOVA

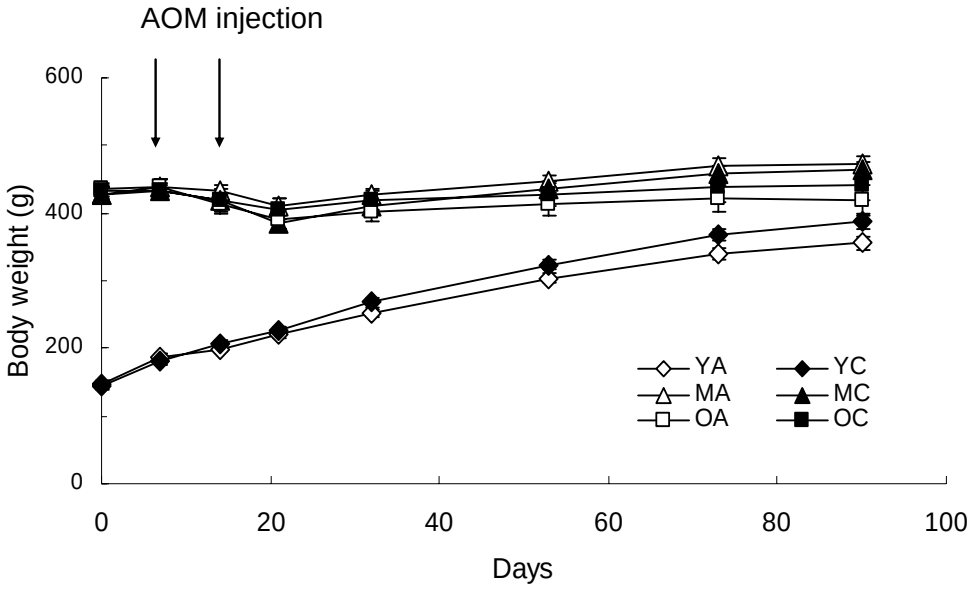

Figure 2. Average body weight (mean ± SE, n=5-6 rats/group). YA: young rats fed AIN (AIN-93 control diet), YC: young rats fed CUR (AIN-93 diet containing 0.6% curcumin), MA: middle-aged rats fed AIN, MC: middle-aged rats fed CUR, OA: old rats fed AIN, OC: old rats fed CUR. Rats were given 2 weekly s.c. injections of AOM (arrow) one week after starting their experimental diets.

2.3.2 Aberrant crypt foci evaluation

Two-way analysis of variance (diet × age) revealed a significant effect of diet ($p < 0.01$) on ACF number, and a significant interaction between diet and age ($p < 0.05$). As shown in Fig. 3, a significant inhibition of ACF by curcumin was observed only in young and old but not in middle-aged rats. The number of ACF in young and old rats was lower in curcumin-fed rats by 49% and 55% respectively ($p < 0.05$). In contrast, there was no difference ($p > 0.05$) in the number of ACF between the two diet groups in middle-aged rats. Age-related differences were also found in the inhibition of different size categories of ACF (Fig. 4). In young rats, the reduction in ACF in curcumin-fed rats was observed mainly in small ACF (2 and 3 multiplicity). In contrast, the numbers of ACF in all size categories were not significantly different in the two diet groups in middle-aged rats. In old rats, there were fewer ACF in all size categories in the curcumin-fed group. The distribution of ACF throughout the whole colon is shown in Fig. 5. The majority of the ACF in old rats were located in the distal to mid-colon in both groups of rats fed CUR and AIN. Distribution of ACF in young and middle-aged rats was similar to that of old rats and was not significantly different between diet groups (data not shown).

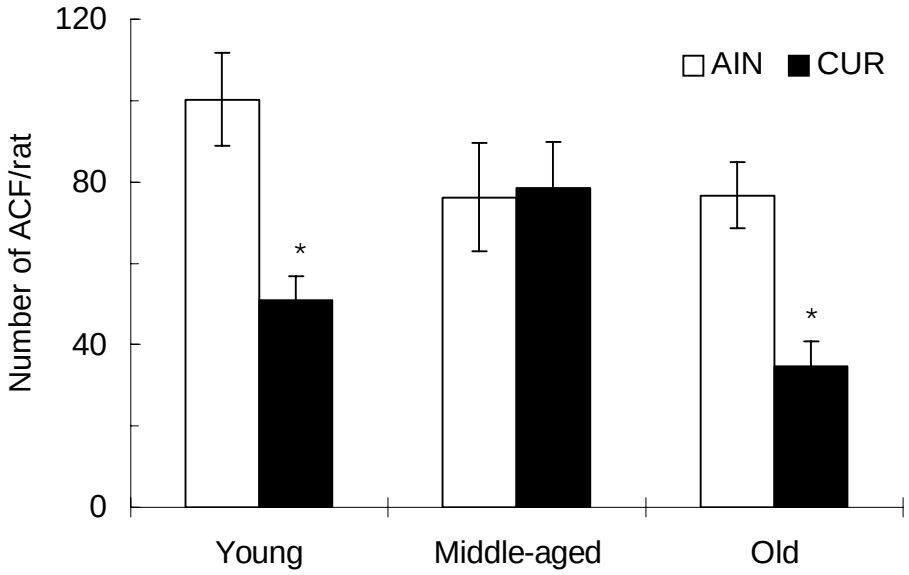

Figure 3. Reduction in number of ACF with 2 and over multiplicity by curcumin in different ages of rats (mean ± SE, n=6 rats/group). AIN is the group fed the AIN-93 control diet and CUR is the group fed the AIN-93 diet containing 0.6% curcumin. * represents significant differences ($p < 0.05$) between AIN and CUR groups in each age group by ANOVA.

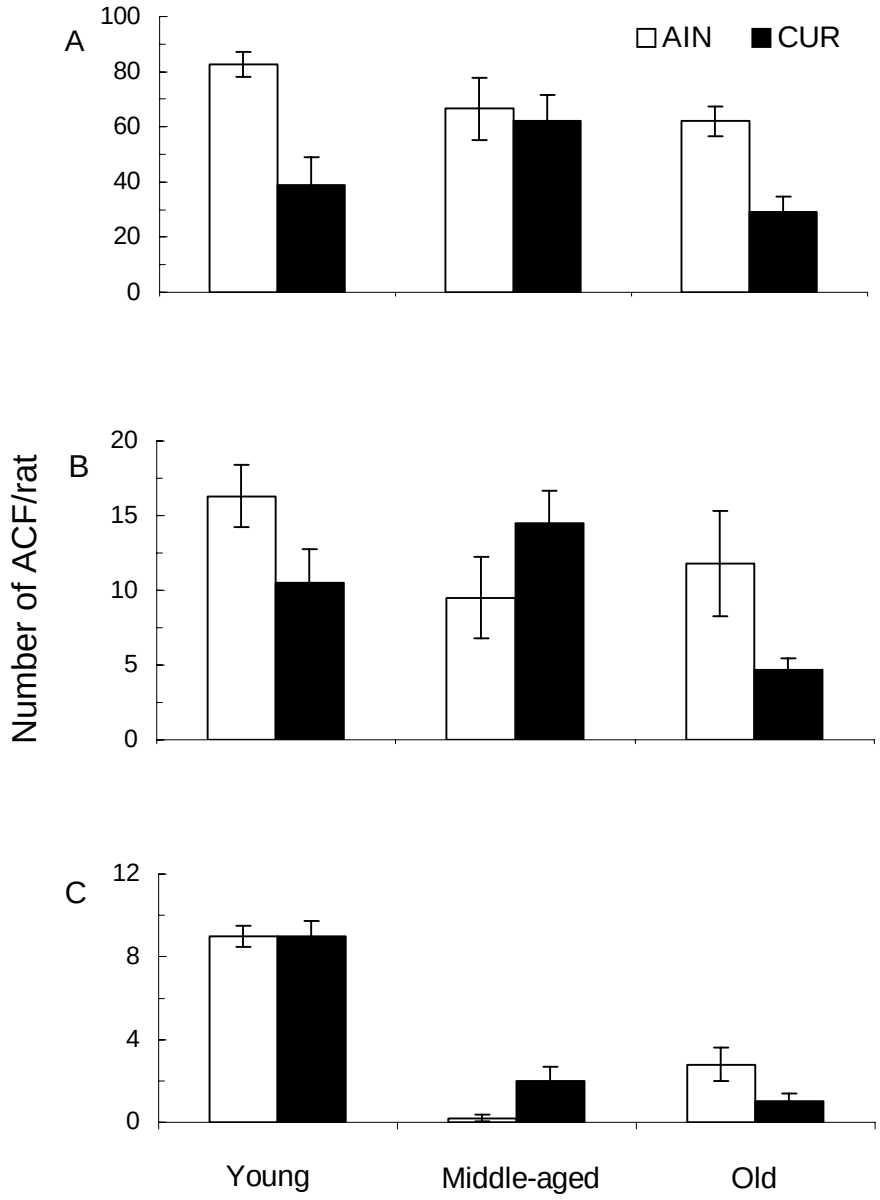

Figure 4. Number of ACF in multiplicity categories of 2 and 3, 4 and 5, and 6 and over in rats fed either the AIN-93 diet (AIN) or the AIN-93 diet containing 0.6% curcumin (CUR) (mean ± SE, n=6 rats/group). A is multiplicity category of 2 and 3 multiplicity, B is multiplicity category of 4 and 5 multiplicity and C is multiplicity category of 6 and over multiplicity.

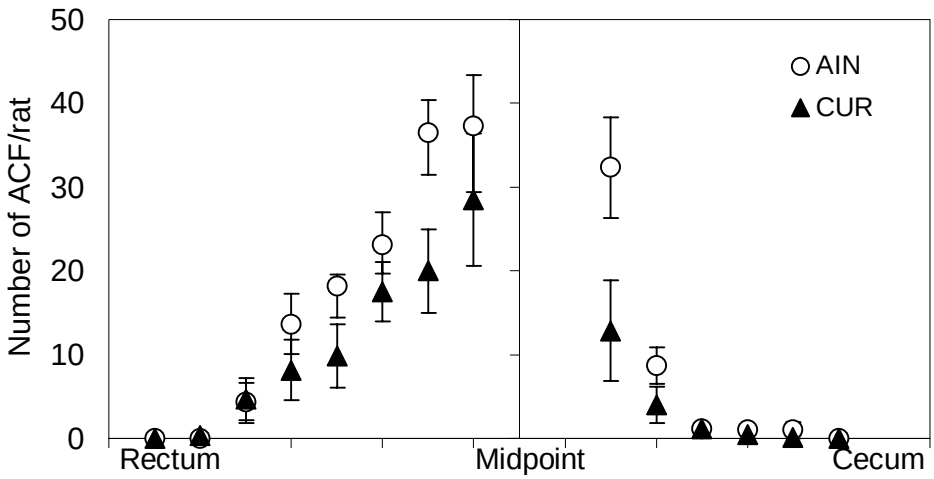

Figure 5. Distribution of ACF in the colon of old rats (mean ± SE, n=6 rats/group) fed either the AIN-93 diet (AIN) or the AIN-93 diet containing 0.6 % curcumin (CUR). Data are depicted for every 2 cm of colon. A 2 cm segment from middle to cecal end was not evaluated for ACF.

2.3.3 Colonic cyclooxygenase-2 immunohistochemistry

The level of COX-2 protein expression in the normal-appearing colon tissue detected by immunostaining was very low in all animal groups (data not shown). Therefore, we were unable to determine conclusively whether COX-2 protein expression in early stage colon carcinogenesis was affected by age or dietary curcumin.

2.3.4 Liver cyclooxygenase-2 mRNA expression

Liver COX-2 mRNA expression levels increased as age increased, in both AIN and CUR groups (Fig. 6). In all age groups, curcumin-fed rats had similarly lower ($p < 0.05$) COX-2 mRNA levels compared to AIN-fed rats: 35% lower in young, 36% lower in middle-aged, and 42% lower in old.

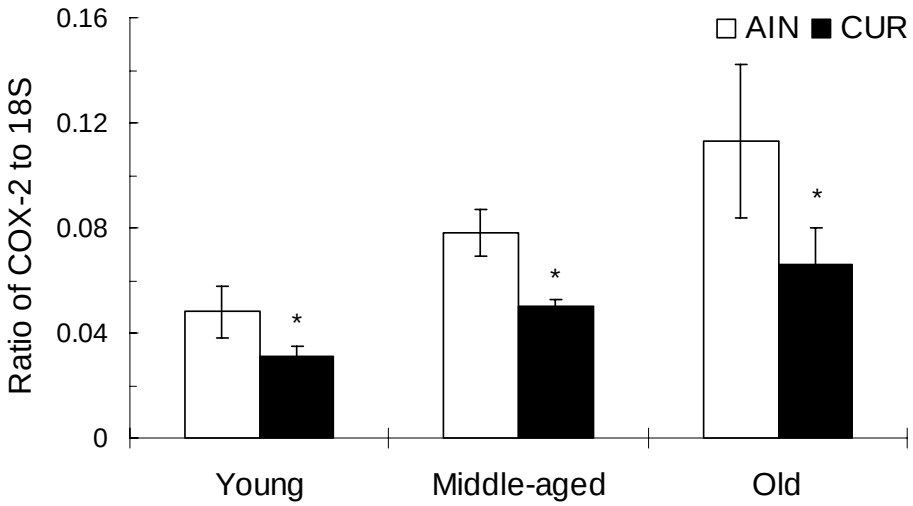

Figure 6. Effect of curcumin on liver cyclooxygenase-2 mRNA expression in relation to 18S mRNA in different aged rats (mean ± SE, n=5-6 rats/group). AIN is the group fed the AIN-93 control diet and CUR is the group fed the AIN-93 diet containing 0.6% curcumin. Cyclooxygenase-2 mRNA expression levels were normalized to expression levels of an internal control, 18S gene. * represents significant differences ($p < 0.05$) between AIN and CUR groups in each age group by ANOVA.

2.3.5 Total serum cholesterol level

Total serum cholesterol levels increased as age increased (Fig. 7). Middle-aged and old rats had higher ($p < 0.05$) total serum cholesterol levels compared to young rats in the control diet group. Cholesterol levels were higher ($p < 0.05$) in young rats fed curcumin than in young rats fed the control diet. In middle-aged and old rats, serum cholesterol levels were not significantly affected by curcumin feeding.

2.3.6 Macroscopic and histopathological observations

Tissues obtained from the study were observed and evaluated by a pathologist. Macroscopic and histopathological changes in different tissues by age or diet are shown in Appendices.

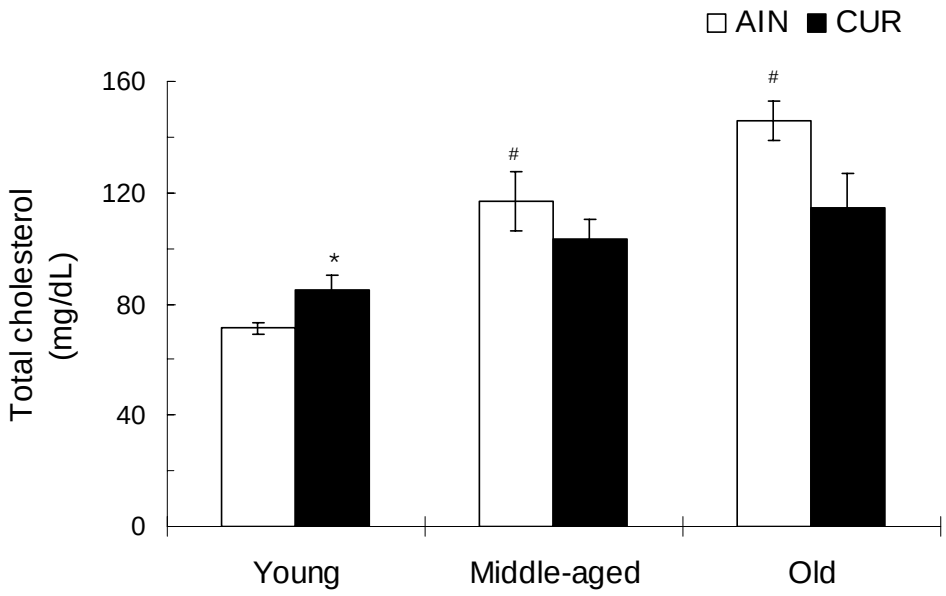

Figure 7. Effect of curcumin on serum total cholesterol levels in different ages of rats (mean ± SE, n=5-6 rats/group). AIN is the group fed the AIN-93 control diet and CUR is the group fed the AIN-93 diet containing 0.6% curcumin. * represents significant differences ($p < 0.05$) between AIN and CUR groups in each age group by ANOVA. $^{\#}$ represents significant differences among different age groups in each diet group by ANOVA.

2.4 Discussion

The most important finding of this study is the significant effect of age on inhibition of the development of early stages of colon cancer by dietary curcumin. A significant age-related difference was found in inhibition of colonic ACF development by 0.6 % dietary curcumin. Rao et al. (71) and Kawamori et al. (72) showed that curcumin effectively inhibits colon tumor in young F344 rats. Interestingly, in this study, curcumin effectively inhibited the number of ACF in young and old rats whereas ACF growth and development in middle-aged rats was not affected by curcumin (Fig. 3). The number of large ACF is more predictive of tumor development than total number of ACF (62). In young rats, feeding curcumin more effectively inhibited smaller ACF than larger ACF. On the other hand, old rats fed curcumin had a much lower number of ACF in all categories. In middle-aged rats, curcumin did not inhibit the number of ACF in any size of category.

It is unclear how curcumin affects progression of small ACF to large ACF. Small ACF (2 and 3 multiplicity) may be more susceptible to curcumin and selected small ACF resistant to dietary curcumin may progress into more advanced stages. Another possible explanation would be that large, advanced ACF might be more resistant to curcumin. The study of Magnuson et al. (62) showed that feeding of cholic acid to AOM-treated rats reduced the number of small ACF, but increased the

growth of large, resistant ACF and the tumor incidence. ACF in rats fed the AIN-76 diet containing 0.2% cholic acid had lower number of apoptotic bodies per 100 cells than ACF in rats fed AIN-76 control diet (62), demonstrating resistance to the cytotoxic effect of cholic acid.

Age also affected ACF development. There was a trend for reduced number of ACF in middle-aged and old rats as compared to young rats fed the control AIN-93 diet in response to the same dose per body weight of AOM. This is in contrast to a previous report (58) in which middle-aged female Sprague-Dawley rats developed a higher number of ACF than did young rats. There are several differences between this study and the previous study including sex, strain and supplier of the rats, as well as diet. In this study, F344 rats were obtained from the NIA aged animal colony at the aged 6 weeks, 12 months and 22 months, and the AIN-93 diet was used as the base diet. The previous study (58) was conducted using female Sprague-Dawley rats from Simonsen Laboratories Inc, aged 4 and 50 weeks and fed standard rat chow. However, the study of Verghese et al. (117), which also used male F344 rats from the NIA colony and the AIN-93 diet, suggested that middle-aged rats were more sensitive to AOM. Variations in development of colonic tumors and ACF due to strain, sex, animal supplier and diet have been reported previously (118). Chung and colleagues (119) recently reported that when C57BL/6JNIA mice were given the same per body

weight dose of AOM, old male mice (21-22 mo) developed significantly more ACF than did young mice (4-5 mo). However, when the same total dose was delivered, young mice developed more ACF, presumably due to the higher dose per body weight. Dix (5) suggested that increase of cancer incidence with age might be caused by change of cell proliferation rate with age. However, Magnuson et al. (58) reported that cell proliferation rate determined by bromodeoxyuridine labeling index was similar between 4 and 50 weeks of age although there was age-related difference in ACF development. Also, Chung et al. (119) showed that age-related susceptibility to AOM is not due to differences of COX-2 expression, cell proliferation, or AOM hydroxylase activity in C57BL/6JNIA mice. At this time, it is not clear whether young animals are more susceptible to AOM-induced colon carcinogenesis than are old animals, or what factors may influence the effect of age.

The precise mechanisms by which age affects response to dietary curcumin are not known. A possible explanation may be differences in the absorption, metabolism, and/or excretion rate of curcumin due to age. Dybing and Soderlund (120) reported decreased metabolism in the very young and old, and proposed that newborns and elderly may be more sensitive to xenobiotics whose toxicity is due to the parent compound. Ireson et al. (111) showed that curcumin metabolites differently inhibited phorbol ester-induced prostaglandin E2 (PGE2) production –

curcumin reduced PGE2 levels to control levels whereas tetrahydrocurcumin, hexahydrocurcumin, and curcumin sulfate had much less inhibitory activity, and hexahydrocurcuminol was inactive. Therefore, the relative amount of curcumin and each curcumin metabolite may affect biological activity of curcumin *in vivo*. However, several studies on the pharmacokinetics of curcumin (121-124) have illustrated that metabolites of curcumin are only detectable in plasma when high dose of curcumin, followed by rapid sampling. Pan et al. (123) reported that when curcumin was administered orally in a bolus dose (1.0 g/kg), curcumin was below detection level within 6 hours and existed as conjugate or reduced form in plasma. Sharma et al. (125) reported curcumin levels in the liver and colon mucosa from female F344 rats fed 2 % curcumin for 14 days. They were able to measure curcumin from liver and colon mucosa but curcumin glucuronide and curcumin sulfate were below detection limit. Therefore, it was unlikely to be able to measure curcumin and all metabolites from tissues of rats fed 0.6% curcumin.

We attempted to estimate the biological activity of curcumin in the three ages of rats using changes in serum cholesterol, COX-2 mRNA in the liver and COX-2 protein expression in the colon. Several studies have demonstrated that curcumin is involved in cholesterol metabolism. Hypocholesterolemic properties of curcumin in male rabbits (69) and in healthy humans who did not have a high fat or high

cholesterol diet (115) have been reported. In this study, curcumin did not reduce total serum cholesterol levels in either middle-aged or old rats. On the contrary, curcumin increased serum total cholesterol in young rats, which had initial lower total serum cholesterol levels than middle-aged and old rats. Most previous animal studies used high fat and high cholesterol diets to investigate the hypocholesterolemic effect of curcumin (69, 126), although a high fat or high cholesterol diet was not applied in the human study where 500 mg of curcumin per day for 7 days reduced total serum cholesterol (115). Therefore, total serum cholesterol levels in the rats in this study may not have been high enough to see hypocholesterolemic effect of curcumin. Harris (127) reviewed the effects of n-3 fatty acids on serum lipid and lipoprotein concentrations in seven species of experimental animals. In all experimental animals, n-3 fatty acids apparently reduced high-density-lipoprotein-cholesterol (HDL) concentrations, which is never observed in humans with fish-oil supplementation. It was suggested that these differences between animals and humans were caused by species differences in lipoprotein metabolism and differences in experimental design (127). Mela et al. (128) also postulated that differences in plasma HDL responses to adiposity in humans and experimental animals would arise by species differences in lipoprotein metabolism. Therefore, changes in total serum cholesterol may not be a good measure of curcumin activity in rats.

Sharma et al. (125) suggested that both colon mucosa and liver are target organs of curcumin as feeding 2 % dietary curcumin for 14 days elevated glutathione-S-transferase levels in the liver and reduced levels of malondialdehyde-deoxyguanosine adduct in colon mucosa. AOM is generally used to induce colon cancer and causes formation of prostaglandins from arachidonic acid as a result of inducing COX-2 expression in the colon (71). Liver is a major organ where not only curcumin but also AOM are metabolized and where prostaglandin formation is induced by AOM (114). Rao et al. (114) reported that curcumin suppressed AOM-induced prostaglandin and thromboxane in the liver and colonic mucosa of young male F344 rats. Therefore, liver COX-2 mRNA and colonic COX-2 protein levels were measured to compare the biological activity of curcumin in the three ages of rats. Liver COX-2 mRNA expression increased as age increased, but curcumin reduced COX-2 mRNA expression to similar extent in three ages of rats, suggesting similar levels of biologically active curcumin in the liver (Fig. 6). This result indicates that age-related differences in the absorption or metabolism of curcumin were not occurred.

As has been reported previously for normal and preneoplastic colonic tissues (46), the levels of COX-2 were not high enough to detect using immunohistochemistry, preventing the determination of whether age or curcumin

reduced colonic COX-2 expression. Therefore, in contrast to the age-related difference in inhibition of colonic ACF by curcumin, any other age-related difference in response to curcumin was not detected. This age-related difference is more likely due to age-related differences in cellular events relevant to colon carcinogenesis rather than in curcumin absorption or metabolism.

In conclusion, these results demonstrate that age affects inhibition of early stage of colon carcinogenesis by curcumin. Further investigations are needed to determine whether aging affects responses to other dietary interventions in colon carcinogenesis or whether this effect is limited to curcumin, and whether age also affects inhibition of advanced tumor development by curcumin. These results suggest that the effect of age should be considered in preclinical study designs of dietary interventions to determine whether age-related differences may be at least partially responsible for lack of correlation of efficacy of dietary interventions in animal and human colon cancer intervention trials.

Chapter 3. Confirmation of lack of preventive effect of curcumin on ACF development in middle-aged rats

3.1 Introduction

Age is a single potent factor contributing to the incidence of cancer, including colorectal cancer. Most of cancers derived from epithelial cells onset at the age of about 40 and after that exponentially increase with age both in men and women (3). Although it is unclear how the aging process is involved in carcinogenesis, the microenvironment of the organism seems to have a significant effect on determining the progression of the malignant phenotype. Successive studies of McCullough et al. (8-10) showed that the development of tumors from liver epithelial cancer cells was affected by changes in tissue microenvironment and that the aging process causes such alterations in the tissue environment. Krtolica et al. (11) also reported that senescent fibroblasts stimulate growth of premalignant cells both *in vitro* and *in vivo*. These findings suggest that the microenvironment can modulate tumor development from initiated cancer cells and aging process may contribute alteration of tissue environment, promoting initiated cancer cells to grow malignant tumors.

The significance of the microenvironment in carcinogenesis led us to hypothesize that the aging process may also affect chemoprevention by dietary

compounds. Interestingly, the previous study showed no inhibition of formation of aberrant crypt foci (ACF), putative lesions of early colon cancer, by dietary curcumin only in middle-aged rats compared to effective reduction in young and old rats (Chapter 2). This loss of preventive activity of curcumin in middle-aged rats was a novel finding, with important implications for human intervention trials. Therefore, it was important to confirm this observation and to further investigate potential mechanisms responsible. Age-related differences in the response in the colon rather than in curcumin metabolism seemed to be responsible for our finding as liver COX-2 mRNA expression, an indicator of biological activity of curcumin, was similarly affected by curcumin in all ages (129).

Curcumin is a polyphenolic compound imparting the yellow color in the spice turmeric, powdered rhizome of *Curcuma logna* Linn. The chemopreventive activity of curcumin in young animal models and in colon cancer cell lines is well established (27, 42, 71, 72, 82-84). However, the chemopreventive activity of curcumin in middle-aged or old animals had not been previously reported.

The anti-inflammatory activity of curcumin has been postulated as a mechanism of colon cancer prevention. In general, COX is a rate-limiting enzyme catalyzing conversion of arachidonic acid into prostaglandins. Two forms, COX-1 and COX-2, are known. COX-1 is constitutively present whereas COX-2 is induced by cytokines,

mitogens, and tumor promoters, and mediates the inflammatory process (86). Many studies have reported that COX-2 is highly expressed in colon tumors (87-89) and inhibition of COX-2 as a mean of colon cancer prevention has been reviewed by Gupta and DuBois (90). Curcumin inhibited phospholipases, responsible for generation of arachidonic acid from membrane phospholipids and formation of prostaglandin E2 (PGE2), both in colon tumors and colon mucosa (71). Curcumin treatment also inhibited COX-2 expression at both mRNA and protein levels in HT-29 colon cancer cells (91). The effect of dietary curcumin on arachidonic acid metabolism has not been investigated in the colon at the stage of ACF formation in either young or older animals. In the previous study (Chapter 2), curcumin inhibited COX-2 mRNA expression in all ages in the liver but the effect of curcumin on colonic COX-2 protein expression estimated by immunohistochemisty was not determined due to very low expression in all groups. Therefore, this study duplicated the ACF study with only middle-aged rats to confirm previous findings of resistance of middle-aged rats to curcumin chemoprevention and investigated if the lack of chemoprevention by curcumin in middle-aged rats is due to a failure of inhibition of COX-2.

This study reports confirmation of the previous findings – middle-aged male F44 rats are resistant to the chemopreventive activity of curcumin against AOM-

induced colon carcinogenesis. Transcriptional levels of COX were lower in the liver but higher in the colon of middle-aged rats fed the curcumin diet. There were no age-related differences in colon COX expression in rats either untreated or treated with AOM. Shortly after AOM treatment, colonic COX-1 mRNA levels were reduced in all ages of rats whereas COX-2 mRNA levels were not affected by AOM in any age group. It should be further studied whether curcumin similarly increases COX expression in the colon of young rats.

3.2 Materials and Methods

3.2.1 Experimental animals and diets

In all the studies, experimental animals were male F344 rats obtained from the colony at the National Institute of Aging (NIA, Bethesda, MD). Young, middle-aged, and old rats were treated at the age of 6 weeks, 12 months, and 22 months, respectively. Control diet was AIN-93 diet (AIN) and experimental diet was AIN-93 diet containing 0.6% curcumin (CUR) prepared by Dyets (Dyets, Inc., Bethlehem, PA). Curcumin was purchased from Sigma (St. Louis, MO).

Experiment 1. Effect of curcumin on colonic ACF formation and arachidonic acid metabolism in the colon and the liver of middle-aged rats: To confirm the previous observation (Chapter 2) of resistance of chemoprevention in middle-aged rats, and to investigate the effect of curcumin on colonic arachidonic acid metabolism, we followed our previous experimental protocol (Section 2.2.1) using only middle-aged rats, and increased the number of animals per group. Twenty middle-aged rats were randomly assigned to either CUR or AIN diets. One week after starting their experimental diets, all the rats were given 2 weekly s.c. injections (15 mg/kg body weight) of azoxymethane (AOM, Sigma) and were maintained on their experimental diets. Three months after the first AOM injection, the rats were killed. Tissue collection and assays are described below. Transcriptional levels of COX-1 and COX-2 were measured in collected colon mucosa and livers. Also, colonic PGE2 levels were evaluated. Two rats fed the AIN-93 diet were found dead 3 days after the second injection of AOM and one rat fed curcumin diet was incorrectly injected with lower dose of AOM. Data from those 3 rats were removed from all analyses.

Experiment 2. Effect of AOM on colonic cyclooxygenase in three different ages of rats: The effect of AOM treatment on the colonic COX-1 and COX-

2 mRNA expression was tested in three different ages of animals. Groups of 12 young, middle-aged, and old male F344 rats were obtained. Six rats in each age group were randomly given a single injection of either AOM (15 mg/kg body weight) or saline after being fed AIN-93 diet for one week. Rats were killed 0 (saline control) or 24 hours after the AOM injection and colons were collected for measurement of COX-1 and COX-2 mRNA levels.

3.2.2 Tissue collections

Colons were immediately placed on a cold surface and scraped to collect mucosa. Half of the mucosa from each rat was fixed in RNAlater (Ambion Inc, Austin, TX) for RNA isolation and the rest was frozen at –80 ^{o}C for the PGE2 immunoassay. Collected livers were stored at –80 ^{o}C until analyzed.

3.2.3 Aberrant crypt foci evaluation

Whole colons were flushed with cold 1 X phosphate buffer saline and opened longitudinally. ACF were evaluated as described in previous studies (Section 2.2.2), (116) from whole colon except for 2 cm of distal colon which was scraped and treated as described above.

3.2.4 Cyclooxygenase-1 and 2 mRNA expression using RT-PCR

Total RNA was isolated from the liver and the colon mucosa using TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was generated using Retroscript kit (Ambion Inc., Austin, TX). An aliquot of cDNA was subjected to RT-PCR for COX-1 or COX-2 genes as described previously (Section 2.2.3). Briefly, gene expression levels of COX-1 and COX-2 were measured using the mouse COX-1 (401 bp) and COX-2 (297 bp) gene-specific Relative RT-PCR Kit (Ambion Inc.), respectively, relative to 18S (498 bp), primer:competimer (1:9). 18S mRNA was used to normalize COX gene expression to control for possible differences in loading. The PCR conditions for COX-1 and COX-2 genes were the same as described in the previous study (Section 2.2.3) with exceptions that an annealing temperature for COX-1 was 61 ^{o}C and the number of thermocycles was 27 and 29 for the colon and the liver cDNAs, respectively. The levels of PCR products of COX-1, COX-2 and 18S were determined using DNA 500 LabChip® and Agilent 2100 bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the ratio of PCR products of COX-1 or COX-2 to 18S were generated.

3.2.5 Prostaglandin E2 using enzyme immunoassay

Frozen colon tissues were homogenized with 50 mM TRIS buffer (pH 7.5)

containing 10 μM indomethacin (MP Biomedicals, Aurora, OH). Colon homogenates were acidified with 2M HCl to pH 3.5 and extracted 3 times with ethyl acetate (10 times of homogenates in volume). Collected ethyl acetate layers following centrifugation at 5,000 g for 5 min were evaporated to dryness under N_2 and reconstituted with the assay buffer for measurement of PGE2 using the prostaglandin E2 enzyme immunoassay kit (Assay Designs Inc., Ann Arbor, MI). Results were expressed as pg PGE2/mg wet tissue.

3.2.6 Data analysis

One-way analysis of variance (ANOVA) was conducted for the all data analyses using SAS software (8.1, SAS Institute Inc, Cary, NC) to determine if there were significant differences between any two groups. Two-way ANOVA was performed to examine the effect of age and azoxymethane treatment and their interaction on the ratio of mRNA levels of colonic COX-1 or COX-2 to 18S.

3.3 Results

3.3.1 Experiment 1. Effect of curcumin on colonic ACF formation and arachidonic acid metabolism in middle-aged rats

Aberrant crypt foci evaluation: As shown in Table 2, there was no significant difference between rats fed curcumin (CUR) and control (AIN) diet in either the total number of ACF or any category of ACF multiplicity. ACF multiplicity is a measure of progression of ACF and is equal to the number of aberrant crypts per focus (62). P-values tended to become close to 1 as multiplicity increased.

Table 2. Number of ACF in different categories of multiplicity in middle-aged rats

Category[a] / Diet[b]	Total	2-3	4-5	6-9	>10
AIN	244.3 ± 18.2	113.4 ± 8.5	34.6 ± 5.6	21.8 ± 6.4	5.3 ± 2.0
CUR	204.1 ± 12.6	95.4 ± 7.7	28.3 ± 2.4	22.9 ± 3.6	5.8 ± 1.2
p – value[c]	0.0937	0.1375	0.3270	0.8799	0.8258

[a] ACF were categorized by their multiplicity

[b] AIN is AIN-93 control diet and CUR is AIN-93 diet containing 0.6% curcumin

[c] There were no significant differences ($p > 0.05$) between diet groups in any category of ACF multiplicity by ANOVA.

Cyclooxygenase mRNA expression and colonic prostaglandin E2 levels:

In the liver, rats fed the curcumin diet had significantly lower ($p < 0.05$) levels of both COX-1 and COX-2 mRNA compared to rats fed the control diet (Fig. 8). On the contrary, colonic COX-2 mRNA levels were significantly higher ($p < 0.05$) in the curcumin diet group compared to the control diet group. Feeding 0.6% curcumin did not significantly affect ($p > 0.05$) either colonic COX-1 mRNA expression (Fig. 8, A) or PGE2 levels (Fig. 8, B). In the liver, transcriptional levels of COX-2 were much lower than COX-1 whereas in the colon, the relative levels of COX-2 to COX-1 mRNA were similar as shown in Fig. 8.

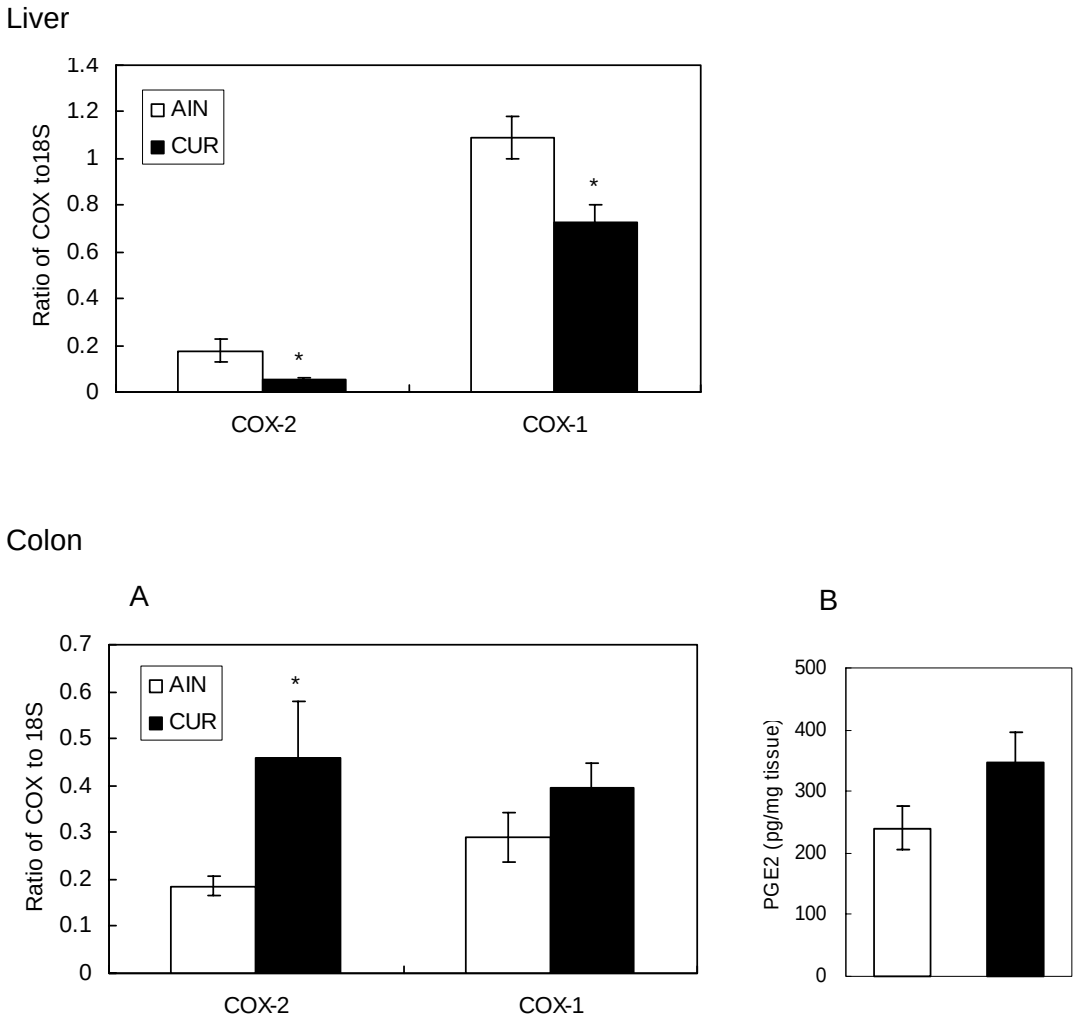

Figure 8. Effect of curcumin on arachidonic acid metabolism in middle-aged rats 3 months after the AOM injection (mean ± SE, n=8-9 rats/group). AIN is the group fed the AIN-93 control diet and CUR is the group fed the AIN-93 diet containing 0.6% curcumin. Transcriptional levels of COX-2 and COX-1 in relation to 18S in the liver (upper) and the colon (lower, A). PGE2 levels in the colon (lower, B). Data values of COX-2 mRNA were log transformed due to large difference in variance between two diet groups. * represents significant differences ($p < 0.05$) between AIN and CUR by ANOVA.

3.3.2 Experiment 2. Effect of AOM on colonic cyclooxygenase mRNA levels in three different ages of rats

Dietary curcumin differentially affected the levels of COX expression in the liver and the colon mucosa of middle-aged rats. The increased colonic COX expression in curcumin-fed rats was unexpected. Therefore, it was investigated whether colonic COX mRNA expression is differently affected by AOM treatment in different aged rats. The levels of colonic COX-1 and COX-2 mRNA were examined in three different ages of rats treated with either AOM or saline.

As shown in Fig. 9, COX-2 mRNA levels were not significantly affected ($p > 0.05$) by AOM treatment in any age group. Two-way analysis of variance (age × AOM treatment) also showed no significant age, treatment, or interaction effects for COX-2. In contrast, in every age group, rats treated with AOM had lower levels of colonic COX-1 mRNA levels 24 hours following injection. When two-way ANOVA was performed, there was a significant AOM effect ($p < 0.05$) whereas neither the effect of age or interaction of age and treatment were significant. The levels of colonic COX-1 and COX-2 were similar among all ages of animals within the same treatment group (Fig. 9). Therefore, transcriptional levels of COX-1, but not COX-2, were inhibited by AOM in all age groups.

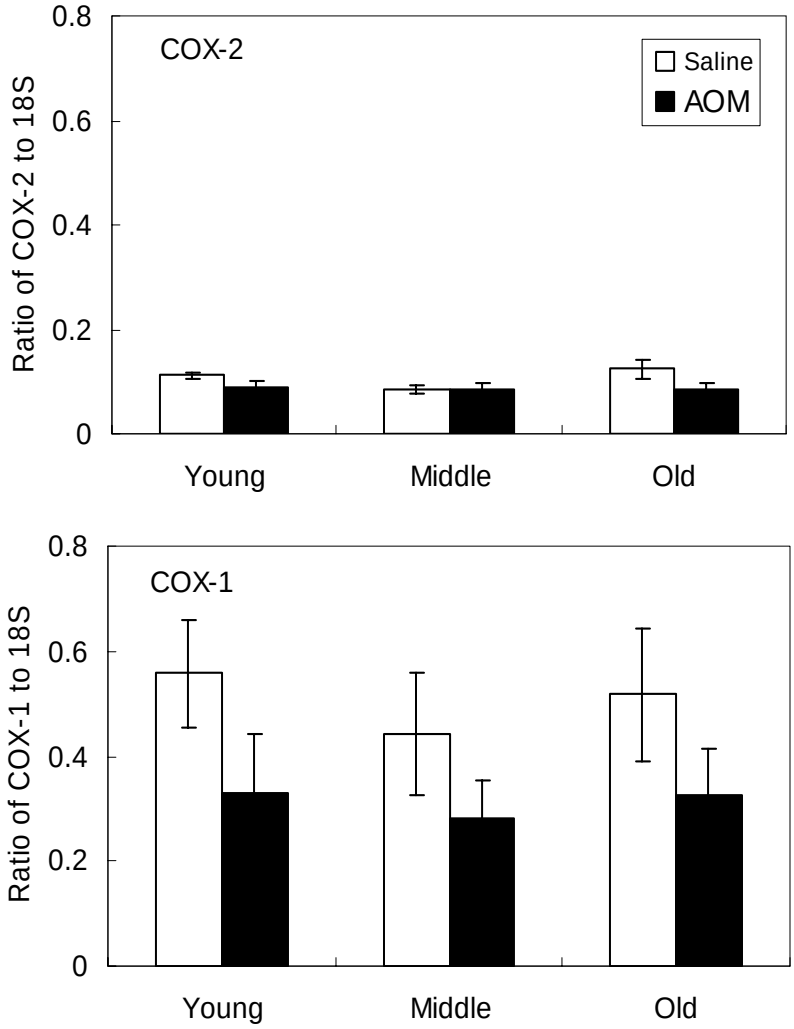

Figure 9. Effect of AOM on colonic mRNA expression of COX-2 (upper) and COX-1 (lower) in relation to 18S expression in three different ages of rats at 0 (saline treatment) or 24 hours after AOM injection (mean ± SE, n=6 rats/group). Two-way ANOVA showed a significant effect ($p < 0.05$) of AOM treatment on colonic COX-1 mRNA expression levels.

3.4 Discussion

This study sought to confirm previous findings of age-related differences in chemoprevention by curcumin and explore possible mechanisms. As described in Chapter 2, the chemopreventive activity of curcumin determined by inhibition of AOM-induced ACF was not observed in middle-aged rats, while significant chemoprevention was observed in young and old rats. Resistance of middle-aged rats to colonic chemoprevention by dietary curcumin is a significant observation. It is middle-aged to older individuals who are targeted for prevention of cancer. However, young animals have been exclusively used in preclinical studies. As shown in Table 2, the number of ACF did not significantly differ by diet group in any multiplicity category of ACF and this was even more apparent in larger ACF (multiplicities of 6-9 and >10), indicating no chemopreventive effect of curcumin. Rats fed curcumin did have significantly lower levels of both COX-1 and COX-2 mRNA in the liver (Fig. 8) as observed previously (Section 2.3.4). Therefore, this study reproduced and confirmed previous findings – middle-aged animals are resistant to the chemopreventive activity of curcumin against AOM-induced colon carcinogenesis.

This study also investigated if arachidonic acid metabolism is potentially involved in the lack of curcumin chemoprevention in middle-aged rats. Previous

studies have suggested that high expression of COX-2 and COX-2-metabolites such as PGE2 are involved in colon carcinogenesis (48, 71, 130, 131). Modulation of arachidonic acid metabolism by inhibiting the COX-2 enzyme is considered an effective mechanism for anticarcinogenic action of chemopreventive agents including curcumin (90). Earlier studies reported that curcumin inhibited the enzyme activity of total COX in the colon mucosa of rats treated with AOM (114) and reduced *ex-vivo* production of PGE2 in AOM-induced colon tumors (71). A more recent study showed that curcumin treatment inhibited COX-2 expression at both mRNA and protein levels in HT29 colon cancer cells which highly express COX-2 (91). However, the effect of dietary curcumin on arachidonic acid metabolism was not investigated in ACF stage of AOM-induced colon carcinogenesis in either middle-aged or young animals. In previous our ACF study (Chapter 2), the poor quality of RNA in formalin-fixed colon tissues made difficult to evaluate COX-2 mRNA expression. Colonic COX-2 protein levels were very low in all age and diet groups as determined by the immunohistochemical staining method. Therefore, in this study, we investigated the effect of curcumin on arachidonic acid metabolism in the colonic mucosa by measuring COX-2 mRNA and PGE2 levels in middle-aged rats. Surprisingly, in contrast in the liver, COX-2 was not inhibited in the colon of middle-aged rats fed curcumin, but significantly higher COX-2 mRNA levels were observed

(Fig. 8A). Inhibition of colonic PGE2 production through inhibiting COX-2 enzyme activity has been targeted for colonic chemoprevention (90) and considered one of the anticarcinogenic mechanisms of curcumin as reviewed above. Thus, the results in the colon gave a rise to the speculation that the lack of inhibitory effect of dietary curcumin on arachidonic acid metabolism might be the cause of failure of colonic chemoprevention by curcumin in middle-aged rats.

However, four lines of evidence indicate the inhibition of ACF formation during initiation and early promotion stage of carcinogenesis by curcumin, a known COX inhibitor and colonic chemopreventive agent, may not be due to inhibition of COX-2 activity and PGE2 production in the colon. First, the enhancing effect of AOM on colonic COX-2 expression or PGE2 production has not been demonstrated during initiation of AOM-induced colon carcinogenesis in spite of general agreement in over-expression of COX-2 and PGE2 in advanced tumors. Colonic PGE2 levels were not significantly affected by two weekly injections of AOM at the stage of ACF formation in mice (132). Takahashi et al. (46) reported that epithelial COX-2 expression was only positive in large adenocarcinoma but not in either dysplastic or hyperplastic ACF, suggesting that the COX-2 expression levels in colonic ACF may be as low as in normal-appearing colon crypts. COX-2 expression increased with advancement of colon cancer in AOM-treated rats (46) and sporadic human cancer

(133). Secondly, it is not evident that inhibition of COX-2 activity and PGE2 production is an effective mechanism to inhibit the initiation of colon carcinogenesis. Administration of rofecoxib, a selective COX-2 inhibitor, for six months to 1,2-dimethylhydrazine-treated rats failed to inhibit ACF formation, but reduced later stage tumor progression (134). Moreover, recent data suggest that many selective COX-2 inhibitors have multiple chemopreventive mechanisms including induction of apoptosis, which might be important in inhibiting the initiation of cancer, in a COX-2 independent pathway (135, 136). Eklou-Kalonji et al. (137) also reported prostaglandin-independent effects of aspirin on cell growth and cell cycle in colon cancer cells. Thirdly, there are no available data demonstrating that curcumin inhibits COX-2 or PGE2 under normal conditions in contrast to an apparent inhibitory effect on highly enhanced COX-2 expression and PGE2 production observed in colon tumors or induced inflammation (70, 111, 113). Lastly, but not least, recent studies described below indicate the importance of COX-2 in maintaining gastrointestinal (GI) homeostasis (138-140). Therefore, COX-2 inhibition may not be an anticarcinogenic mechanism during initiation of colon carcinogenesis and thus may not be responsible for the resistance to curcumin chemoprevention in middle-aged rats.

In general, COX-2 expression is low under normal conditions and rapidly induced in response to inflammatory signals whereas COX-1 is constitutively

expressed in various normal tissues including the GI mucosa. These differences in the COX isoforms lead to the idea that COX-1 has a housekeeping role in GI mucosa. As a result, inhibition of COX-1 but not COX-2 was considered to be responsible for the GI damage mainly due to deficiency of prostaglandins as seen in the use of non-steroidal anti-inflammatory drugs (NSAID). However, recent studies using different types of COX inhibitors have shown that inhibition of not only COX-1 but also COX-2 is required for NSAID-induced GI injury (139, 140). The administration of a conventional NSAID such as indomethacin causes hemorrhagic damage in the small intestine of rats (139) and small bowel ulcer in mice (140). The combined administration of the COX-1 selective inhibitor and the selective COX-2 inhibitor induces such damage in the small intestine of rats (139) and mice (140). However, the administration of either the selective COX-1 inhibitor alone or the selective COX-2 inhibitor alone does not produce such damages (139, 140). The study of Sigthorsson et al. (140) further supports the importance of COX-2 in maintaining integrity of GI mucosa, showing occurrence of an ileal ulcer that is distinguished from the ulcer caused by indomethacin in long-term COX-2 deficiency. MacNaughton and Cushing (141) also reported constitutive expression of COX-2 mRNA and protein in mouse colon. Therefore, these studies suggest that both COX-1 and COX-2 play roles in maintaining the levels of prostaglandins required to ensure the integrity of GI

mucosa.

In spite of recent researches emphasis on modulation of arachidonic acid metabolism in colonic chemoprevention, there were no reports on the effect of AOM on COX-2 expression in the colon. Experiment 2 was conducted to determine whether colonic COX-1 and COX-2 mRNA expression is affected by the initiation of AOM-induced carcinogenesis in three different aged rats. Basal COX-1 mRNA levels were similar among different ages of rats, and COX-1 mRNA expression was similarly inhibited shortly after AOM injection in all age groups (Fig. 9). Also, curcumin alone does not seem to affect colonic COX-2 mRNA levels. Feeding 0.6% curcumin for 10 days did not increase COX-2 mRNA expression in any age of rats not treated with AOM (unpublished observations). This fact excludes the possibility that feeding curcumin increases basal levels of COX-2 mRNA expression only in the colon of middle-aged rats. Therefore, effect of curcumin on arachidonic acid metabolism after AOM treatment should be examined in young rats in order to determine if failure of inhibition of COX-2 mRNA expression and PGE2 production in the colon contributes to the lack of chemoprevention by curcumin in middle-aged rat.

In summary, in the middle-aged group, rats fed curcumin had higher COX-2 mRNA levels in the colon in contrast to reduced COX mRNA levels in the liver.

AOM decreases COX-1 mRNA expression, and does not enhance COX-2 mRNA expression immediately following injection regardless of the ages of animals. Moreover, recent data do not support the contention that modulation of arachidonic acid metabolism plays an important role in preventing initiation of colon carcinogenesis. Whether curcumin increases COX-2 mRNA expression in young rats as in middle-aged rats should be investigated during early stage of AOM-induced carcinogenesis to determine if arachidonic acid metabolism is involved in the resistance of middle-aged rats to curcumin chemoprevention.

Chapter 4. Effect of AOM and curcumin on transcriptional induction of Hsp70

4.1 Introduction

Curcumin did not inhibit the formation of colonic ACF in middle-aged rats, despite effective reduction of ACF in young and old (Chapter 2). It is important to understand the underlying mechanism(s) responsible for resistance to curcumin chemoprevention in middle-aged animals as it is middle-age to older human population who are not only likely suffering from colorectal cancer but also targeted for dietary chemoprevention.

Cyclooxygenase (COX) enzymes are known to be rate-limiting enzymes in production of prostaglandins from arachidonic acids. Many epidemiological, clinical, and experimental studies (142-146) have reported a colonic chemopreventive effect of nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit COX activity (147). In support of this implication of preventive effect of NSAIDs in colon cancer, high levels of COX-2 expression and PGE2 were reported in AOM-induced colon tumors as well as in familial adenomatous polyposis (FAP) and sporadic colon tumors (88, 148). However, long-term use of traditional NSAIDs induces deleterious damage in the gastrointestinal mucosa probably due to reduced levels of

prostaglandins by inhibition of both COX-1 and COX-2 (139, 149). Therefore, COX-2 specific inhibitors are attractive in chemoprevention of colon cancer.

Curcumin is also known to be a COX inhibitor, although it is unclear whether curcumin is COX-2 specific or an inhibitor of both COX-1 and COX-2. Zhang et al. (113) reported that curcumin specifically inhibited the expression of COX-2 without affecting the level of COX-1 in HT-29 colon cancer cells. On the contrary, curcumin more effectively inhibited peroxidase activity of COX-1 compared to COX-2 *in vitro* (68). A previous study (71) reported involvement of arachidonic acid metabolism in colonic chemoprevention by curcumin. However, there is little evidence to suggest that modulation of arachidonic acid metabolism by curcumin is responsible for the age-related difference in inhibition of colonic ACF formation. Available data do not support that COX-2 or PGE2 levels are elevated in initiation of colon carcinogenesis, the stage of ACF formation or earlier. AOM treatment did not significantly affect the PGE2 levels in the colon of mice at the stage ACF form in either high-fat or low-fat diet group (132). In the previous study (Section 3.3.2), colonic COX-2 mRNA expression was not affected by AOM shortly after the treatment. On the contrary, COX-1 mRNA levels were reduced in the colons of AOM-treated rats.

The elevation of colonic levels of COX-2 or PGE2 seems to be more related to progression of colon carcinogenesis. COX-2 expression increased with

advancement of colon cancer in AOM-treated rats (46) and sporadic human cancer (133). Moreover, rofecoxib, a selective COX-2 inhibitor failed to inhibit ACF formation and only effectively reduced tumor progression (134). In addition, age-related differences were not observed in colonic COX-1 or COX-2 mRNA expression levels before or after AOM treatment (Section 3.3.2) whereas basal liver COX levels appeared to increase in older animals (data not shown).

AOM has been widely used to induce colon tumors in preclinical models. We do not know why AOM-induced tumors mainly occur in the colon. It is also still unclear what specific enzymes are involved in metabolic activation of AOM. Several studies suggested that metabolic activation of AOM is initiated by hepatic CYP2E1 (35). However, administration of diallyl sulfide, a CYP2E1 inhibitor, increased AOM-induced formation of ACF in F344 rats (150). Moreover, initial levels of colonic or liver DNA methyl adduct after the AOM treatment did not predict the differential susceptibility to colon tumors in inbred mice, and both susceptible and resistant mice strains had equivalent metabolic capacity estimated by production of O^6-methyl guanine after the incubation of colon microsomes with AOM (151). Also, several studies indicated that neither DNA repair enzyme activity or cell proliferation rates in the dimethylhydrazine (DMH)- or AOM-treated colons are modulated by dietary chemopreventive agents (152, 153) or aging (58, 119). Dietary calcium

(152) or fish oil (153) did not affect either O^6-alkyl guanine alkyltransferase activity or cell proliferation after DMH or AOM treatment in the same models where they showed chemopreventive activity and decreased levels of mutations or DNA adducts. Therefore, it was difficult to reason that any of AOM metabolism, repair enzyme activity, or cell proliferation was involved in age-related differences in curcumin chemopreventive efficacy.

Apoptosis plays a role in maintaining the number of cells in normal mitotic tissues such as the colon. Apoptosis is also an important mechanism to remove unwanted cells due to severe DNA damage (97). Induction of apoptosis right after carcinogen treatment occurs in conjunction with activation of DNA repair systems (154), and therefore apoptosis may be an important mechanism to reduce mutation load and prevent initiation of cancer.

Many different proteins regulate apoptosis. Heat shock proteins (HSP) induced by stress may function at key regulatory points in the control of apoptosis (155). Hsp70 has been most studied in terms of its transcriptional regulation and relationship with apoptosis. Hsp70 is induced by exposure to various physiological and environmental stresses including elevated temperature, heavy metals, amino acid analogs, and oxidative stress (156). It was also recently reported that occupational exposure to coke-oven emission, which is genotoxic, increases Hsp70 protein levels

in lympocytes in exposed workers (157). Induced Hsp70 interacts with proteins damaged by stress and restores them to function properly allowing cells to maintain their function and survive (156). On the other hand, overexpression of Hsp 70 inhibits cell death, perhaps in spite of severe damage, and therefore increases the chance that damaged cells will grow into tumors. Hsp70 protects cells from both stress-induced caspase-dependent and –independent apoptosis (101, 102).

Transcription of Hsp70 is highly regulated through the transcriptional factor, heat shock factor 1 (HSF1), in eukaryotes (156). Transcriptional activation and attenuation of Hsp70 in response to stress shows how strikingly these processes are regulated so that they rapidly respond to differing intensities and sources of stress and prevent overexpression. Hsp70 induction also seems to be affected by maturation and/or the aging process (158-162). There is accumulating evidence that transcriptional activation of Hsp70 by stress is delayed and/or decreased in older animals because of lower HSF1 binding activity to DNA (163).

Interestingly, curcumin treatment induces Hsp70 under stress conditions (164) probably by increasing HSF1 binding activity to DNA, directly opposite to the behavior of HSF1 in older individuals. Therefore, this study hypothesized that when AOM produces methylated adducts in association with DNA, RNA, and protein, it may induce Hsp70 and that curcumin may increase induction of Hsp70 by AOM

under this stress condition where transcription of Hsp70 is triggered. This preliminary study was conducted to investigate whether AOM induces Hsp70 expression and whether curcumin increases AOM-induced Hsp70 expression in the colon and the liver.

4.2 Materials and Methods

4.2.1 Experimental animals and diets

Experimental animals were male 4-5 week old F344 rats. Rats were randomly assigned into 8 groups (3 or 4 rats per group) depending on their experimental diets and time killed. Among 31 rats, 8 rats were fed AIN-93 diet containing 0.6% curcumin and the rest were fed AIN-93 control diet. Except for rats in the untreated control group (UNT), remainder (n = 4/group) was administered a single s.c. injection of AOM at the dose of 15 mg/kg body weight one week after being fed their experimental diets. Rats in the control diet group (AIN) were killed 1, 3, 7, 14, and 28 days after AOM injections. Rats fed the curcumin diet (CUR) were killed 3 (AOM-3DAY) and 14 days (AOM-14DAY) after the AOM injections. Mucosa was scraped from colons for isolation of RNA and immunoassay. Livers were also dissected and frozen using liquid nitrogen. Collected tissues were stored

at –80 ^{o}C until analyzed.

4.2.2 Gene expression levels using RT-PCR

Total cellular RNA from colonic mucosa was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using Retroscript kit (Ambio Inc, Austin, TX) as described above (Section 2.2.3). COX genes were amplified using mouse COX-1 or COX-2 gene-specific Relative RT-PCR Kit (Ambion Inc.). The sequences for the specific oligonucleotides for Hsp70 were as previously described (165), and for p21 were as follows (sense: AGC AAA GTA TGC CGT CTC T, antisense: GAG TGC AAG ACA GCG ACA AG, Invitrogen). An aliquot of cDNA was subjected to amplification for each gene using specific oligonucleotides. Ribosomal gene 18S (498 bp) was used as an internal control. PCR reactions for mRNA amplification of each gene were carried out using the DNA engine (MJ Research, Waltham, MA). The same thermocycling conditions were used as in COX-2 amplification (Section 2.2.3) except that the number of cycles was 28 for Hsp70 and 26 for p21 and annealing temperatures were 59 and 60 ^{o}C, respectively. The PCR products from multiplex reactions were quantified using DNA 500 LabChip® and Agilent 2100 bioanalyzer as described previously (Section 2.2.3).

4.2.3 Colonic PGE2 levels using EIA

Colonic PGE2 levels were measured as described above (Section 3.2.5). Data values were presented as pg of PGE2 per mg of wet tissue.

4.3 Results

4.3.1 Hsp70 mRNA expression in the colon

Fig. 10 shows changes in Hsp70 mRNA expression in the colon with the time after AOM injections. Colonic Hsp70 mRNA tended to decrease for 3 days after AOM injections and after that increased to the level of AOM-untreated controls (Day 0). Curcumin feeding did not significantly alter colonic Hsp70 mRNA levels as compared to rats fed the control diet (Fig. 10).

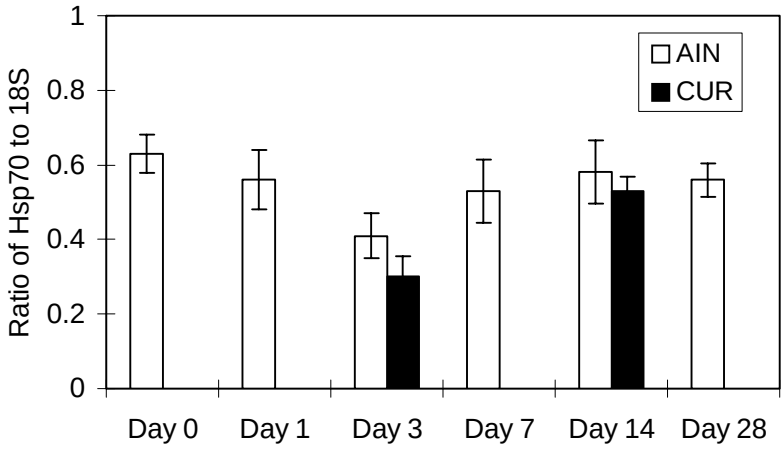

Figure 10. Time course of Hsp70 mRNA expression in relation to 18S mRNA expression in the colon of young rats following AOM injection (mean ± SE, n=3 or 4 per group). Rats in Day 0 were AOM-untreated control. Rats in Day 1 were killed 1 day after AOM injection vice versa. AIN: AIN-93 control diet, CUR: AIN-93 diet containing 0.6% curcumin

4.3.2 Hsp70 mRNA expression in the liver

As shown in Fig. 11, liver Hsp70 mRNA levels were similar to basal levels (Day 0) for at least 7 days following AOM injection. At Day 14 and 28 time points, Hsp70 mRNA levels were higher than levels at the Day 0. Rats fed curcumin had similar Hsp70 mRNA levels when they were compared to control diet group at the same time points (Fig. 11).

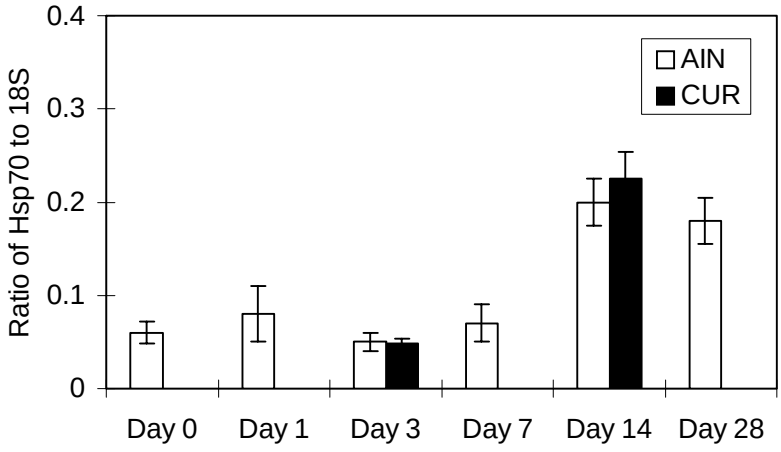

Figure 11. Time course of Hsp70 mRNA expression in relation to 18S mRNA expression in the liver of young rats following AOM injection (mean ± SE, n=3 or 4 per group). Rats in Day 0 were AOM-untreated control. Rats in Day 1 were killed 1 day after AOM injection vice versa. AIN: AIN-93 control diet, CUR: AIN-93 diet containing 0.6% curcumin

4.3.3 p21 mRNA expression in the colon

Colonic mRNA levels of p21 fluctuated over the experimental time points and did not appear to be affected by AOM (Fig. 12). At the Day 3 time point, p21 mRNA levels of rats fed control diet were significantly higher ($p < 0.05$) than those of rats fed the curcumin diet. However, p21 mRNA levels in curcumin-fed rats were only measured at one time point. Due to fluctuation of transcriptional levels of p21 with time in the control diet group, it is inconclusive whether curcumin affected p21 transcriptional levels in this study.

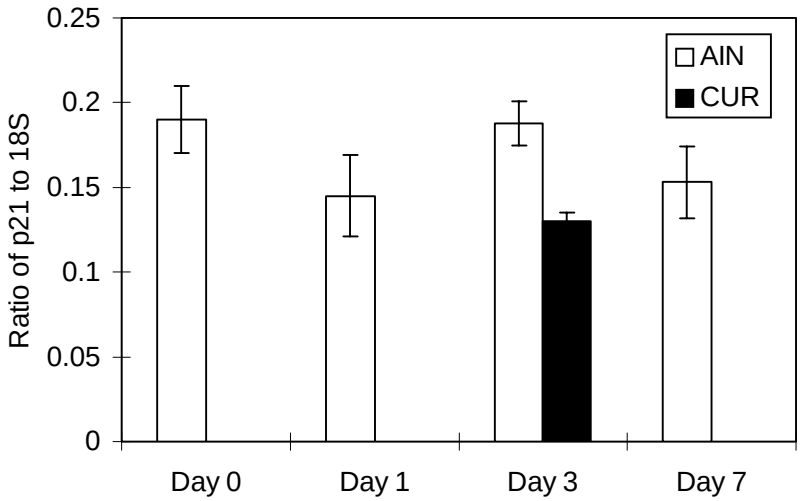

Figure 12. p21 mRNA expression in relation to 18 mRNA expression in the colonic mucosa of young rats following AOM injection (mean ± SE, n=3 or 4 per group). Rats in Day 0 were AOM-untreated control. Rats in Day 1 were killed 1 day after AOM injection vice versa. AIN: AIN-93 control diet, CUR: AIN-93 diet containing 0.6% curcumin

4.3.4 COX-1 and COX-2 mRNA expression in the colon

The levels of colonic COX-2 mRNA in AOM-treated rats (AOM-3DAY and AOM-14DAY) were similar to AOM-untreated rats (AOM-UNT) among groups fed the control diet (Fig. 13A). In rats fed the control diet, COX-2 mRNA levels did not change with time up to 28 days after AOM injection (data not shown). A significant effect of feeding of curcumin on the levels of colonic COX-2 mRNA was not evident 3 days after AOM injection (AOM-3DAY). However, 14 days after the AOM injection (AOM-14DAY), rats fed the curcumin diet had significantly higher ($p < 0.05$) colonic COX-2 mRNA expression (Fig. 13, A) and PGE2 levels (Fig. 13, B) compared to rats fed the control diet.

In contrast to COX-2 mRNA levels, AOM-treated rats (AOM-3DAY) in both diet groups had significantly lower ($p < 0.05$) COX-1 mRNA levels than UNT rats (Fig.13A). Fourteen days after the AOM injection (AOM-14DAY), colonic COX-1 levels returned to basal levels in both diet groups. However, COX-1 mRNA levels recovered to a greater extent in curcumin-fed compared to control-fed rats, resulting in a significantly higher ($p < 0.05$) COX-1 mRNA expression in curcumin diet group compared to control diet group in AOM-3DAY (Fig. 13A). There were no significant differences in the levels of liver COX-1 or COX-2 mRNA expression regardless of diet, AOM treatment, or exposure time (data not shown).

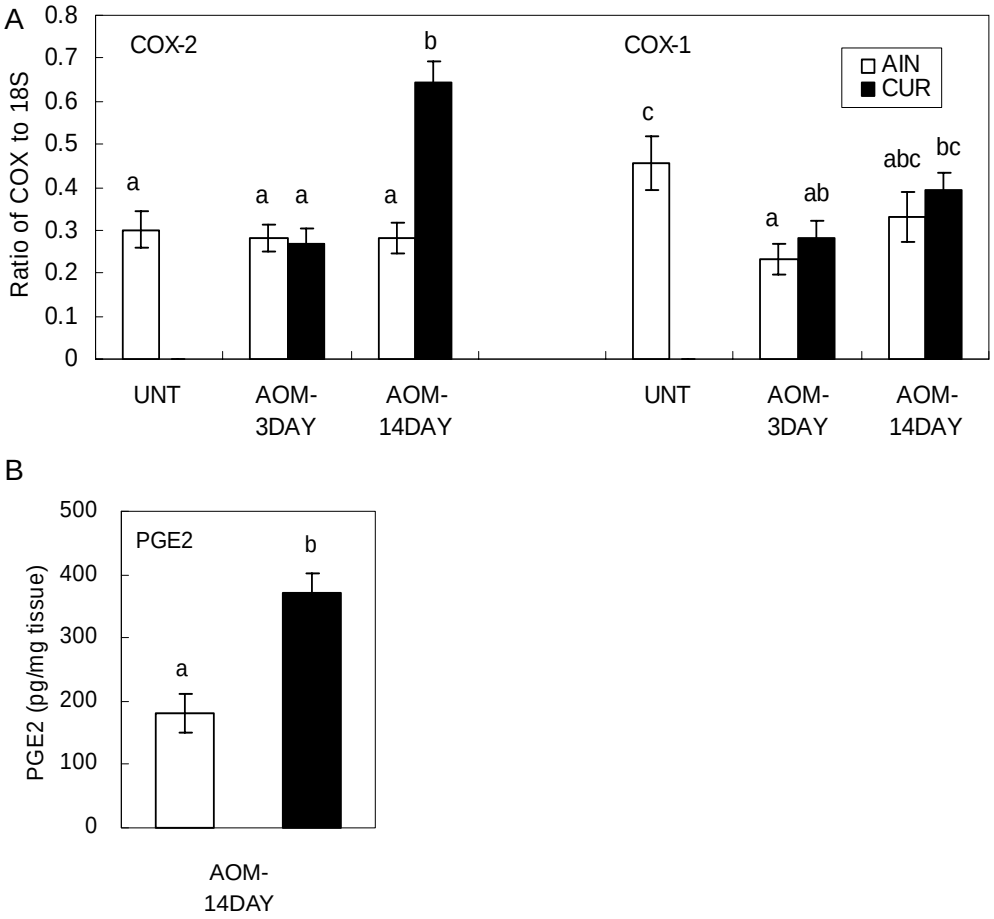

Figure 13. Effect of curcumin on colonic arachidonic acid metabolism in young rats (mean ± SE, n=3 (UNT) or 4 rats/group). Transcriptional levels of COX-2 and COX-1 in relation to 18S (A). PGE2 levels at AOM-14DAY (B). UNT: rats fed control diet (AIN) for one week and killed; AOM-3DAY: rats injected with AOM after feeding curcumin (CUR) or control diet (AIN) for one week and killed 3 days after the AOM injection; AOM-14DAY: rats injected with AOM after feeding of CUR or AIN for one week and killed 14 days after the AOM injection. There were no significant differences ($p > 0.05$) by ANOVA among groups bearing the same alphabets.

4.4. Discussion

Hsp70 mRNA levels in the colon were not elevated after AOM injection. Rats fed curcumin have not increased colonic Hsp70 mRNA levels compared to rats fed the control diet. These results were unexpected as it was hypothesized that AOM would induce Hsp70 expression and that this induction would be further enhanced by curcumin. One possible explanation is that Hsp70 induction occurred before the 24 hour time point. If AOM can induce the heat shock response, the time course of Hsp70 expression may resemble, but be slightly earlier than, the time course of apoptosis induction. Previous studies show that the apoptotic incidence reached maximum levels at 6~8 hours after AOM injection (153) and cellular rate of apoptosis was almost back to basal levels 3 days after AOM injection in young rats (166). In our study, the transcriptional level of Hsp70 was lower after AOM injection, although this was not significant, possibly because there were only 3 or 4 rats per group. Hsp70 mRNA levels are regulated by Hsp70 protein by a negative feedback mechanism (167). It is possible that Hsp70 protein expression was induced by the AOM injection and was present prior to apoptosis induction (i.e. earlier than the peak of apoptosis, 8 hours after AOM injection). If that occurred, then Hsp70 mRNA may be already attenuated by high levels of Hsp70 protein at 24 hours following AOM injection, which was the time it was measured.

Another possible explanation of the lack of effect of AOM and curcumin on Hsp70 may be involvement of other potential Hsp70 effectors. CO_2 was used for euthanasia of rats and variation in the time for rats to succumb to CO_2 might be reflected in the results as transcription of Hsp70 can be induced by hypoxic conditions (168). If this is the case, Hsp70 mRNA levels of rats untreated with AOM do not represent basal levels and the effect of AOM and curcumin on Hsp70 expression level cannot be detected. Also, AOM untreated control animals were not included at each time point, therefore the effect of the developmental stage of rats on Hsp70 expression cannot be assessed, making it difficult to interpret the increase of Hsp70 transcriptional levels at 7 days after AOM injection in the liver.

Transcriptional levels of p21 were measured in the colon in order to estimate the extent of AOM-induced DNA damage, as p21 levels are known to increase in response to DNA damage. However, our study did not show that AOM affects p21 mRNA expression. Aizu et al. (169) suggested that p21 expression was not affected by AOM in the mouse colon both at transcriptional and translational levels due to down-regulation of p300, the transcriptional co-activator of p53 by AOM. Their study showed that AOM increased both transcriptional and translational levels of p21 and other p53-regulated genes such as Bax and Gadd45 3 hours after AOM injection in the liver whereas AOM did not affect expression of those genes in the colon –

transcriptional levels were not changed when measured at 0, 3, 6, 24 and 48 hours after AOM injection. Therefore, in this study where AOM was used as a DNA damaging agent, p21 mRNA levels may not be a good indicator of DNA damage with time.

In the previous study (Section 3.3.1), feeding 0.6% curcumin did not inhibit either COX mRNA expression or PGE2 production in the colon, but rather increased COX-2 mRNA levels. This lack of inhibitory effect of dietary curcumin on arachidonic acid metabolism might be the cause of failure of colonic chemoprevention by curcumin in middle-aged rats. However, as reviewed above, it is also now questionable whether inhibition of arachidonic acid metabolism is an important chemopreventive mechanism in the early stage of colon carcinogenesis. Based on recent reports, another possibility is that the increase of COX-2 mRNA expression in curcumin-fed rats was a biologically required event. Tanaka et al. (138) reported that COX-2 mRNA levels were up-regulated in the stomach shortly after the administration of a selective COX-1 inhibitor but not after a selective COX-2 inhibitor. A selective COX-1 inhibitor decreased PGE2 content in the stomach mucosa 2 hours after administration of the inhibitor however, levels were being recovered by 8 hours due to an increased expression of COX-2 (138). This suggests a compensatory role of COX-1 and COX-2 in maintaining the PGE2 content in

gastrointestinal mucosa. We investigated if the increased COX-2 mRNA in the curcumin diet group was associated with reduced COX-1 expression. Feeding 0.6% curcumin to young rats did not affect COX-2 mRNA expression 3 days after AOM treatment (Fig. 13A). However, similar to the previous result in middle-aged rats, significantly higher levels of COX-2 mRNA were measured 14 days after AOM treatment in the colonic mucosa of young rats fed curcumin compared to rats fed the control diet. Colonic levels of PGE2 were also significantly higher in young rats fed the curcumin diet. This was not consistent with a previous study by Rao et al. (114) showing a significant reduction in colonic production of PGE2 after feeding young rats a 0.2 % curcumin diet for 19 days. However, in their study, COX activity was measured as the *in vitro* production rate of prostaglandins including PGE2 by colonic microsomal proteins in the presence of sufficiently added arachidonic acid whereas in our study, endogenous PGE2 levels were measured in the colonic homogenates. Recent reports suggest that phospholipase activity is reduced in both AOM-induced colonic ACF and tumors, reducing available free arachidonic acid (170). Therefore, *in vitro* measurement of PGE2 in the previous study might not properly reflect the production rate of endogenous prostaglandins *in vivo* if AOM-induced carcinogenesis reduces available arachidonic acid.

The increase of COX-2 and PGE2 in young rats fed the curcumin diet seems

to be related with decreased COX-1 mRNA levels in the colon, shortly after the AOM injection. The elevation of COX-2 mRNA expression at 14 days was preceded by the reduction in COX-1 mRNA levels 3 days after AOM injection (Fig. 13A). Although one cannot directly compare two studies, which differ in age of animals and duration of study, we speculated that the higher COX-2 mRNA levels observed previously at the stage of ACF formation in middle-aged rats fed curcumin diet (Section 3.3.1) might be also due to a response to decreased COX-1 mRNA levels by AOM treatment. The previous study (Section 3.3.2) demonstrated that the inhibitory effect of AOM on colonic COX-1 mRNA was not limited to young animals. Therefore, aging does not appear to alter the effect of curcumin on colonic COX-2 mRNA expression in either AOM-treated or untreated rats.

In conclusion, transcriptional levels of Hsp70 were not significantly affected by either AOM treatment or curcumin probably because transcriptional induction of Hsp70 occurred earlier than our first time of measurement at 24 hours. p21 also did not appear to be significantly affected by AOM treatment. Rats fed curcumin had significantly increased transcriptional levels of COX-2 and PGE2 levels in the colon of young rats, which probably was a compensatory mechanism in response to initial reduced COX-1 mRNA expression. Therefore, this study and the previous study in Chapter 3 together suggest that the effect of curcumin on arachidonic acid metabolism

in the colon may not contribute to the age-related difference in the chemopreventive activity of curcumin.

Chapter 5. Effect of aging on AOM-induced apoptosis in the colon of rats

5.1 Introduction

Apoptosis is genetically programmed cell death characterized by a highly ordered decomposition of DNA and proteins (171). Regulation of cellular apoptosis is important for tissue homeostasis. Inappropriate activation of apoptosis may cause loss of constituent cells and result in tissue dysfunction, or tissue may retain cells prone to mutations and potentially develop neoplasia, especially if cells are highly resistant to apoptotic stimuli. The aging process might be derived by an altered apoptotic response (172, 173). It is becoming evident that deregulation of apoptosis may cause age-related diseases including neurodegenerative diseases, osteoarthritis, cardiovascular diseases, and cancer (173, 174).

It is generally accepted that cellular dysfunction is related with loss of cells due to a high incidence of apoptosis in some post-mitotic tissues like neurons and muscles (174). However, how the aging process affects the regulation of apoptosis in mitotic tissues has been understudied. In the normal colon, age does not appear to significantly affect either cell proliferation or apoptosis (109). Lee et al. (109) speculated that age-related differences in apoptosis may not be sufficient enough to

detect, as apoptosis rarely occurs both in young and old normal colonic epithelium. Apoptosis may more likely take place upon exposure to toxic substances such as carcinogens, and cellular control of apoptosis in response to carcinogens may significantly affect not only carcinogenesis but also cellular function in the colon.

Azoxymethane (AOM) has been widely used to induce colon cancer in rodents. AOM is metabolized to highly reactive methyldiazonium, which can bind to DNA and cause mutations (35). In mediating cellular responses to the DNA damage, transcriptional factors, p53, AP-1 (activator protein-1), and nuclear factor kappa B (NF-κB), play significant roles (175). In contrast to the p53 tumor suppressor, which induces cell cycle arrest and apoptosis, activation of NF-κB promotes resistance to programmed cell death (176). Paradoxically, many DNA damaging agents induce activation of both p53 as well as NF-κB (176).

In most type of cells, inactive NF-κB is sequestered in cytoplasm through interaction with the inhibitory proteins, IκBs (175). Upon exposure to stimuli such as cytokines, reactive oxygen species, and genotoxic drugs, IκBs are phosphorylated by their kinase, IKK, and rapidly degraded, allowing NF-κB to be released and translocate to the nucleus where it subsequently activates its target genes. Many of NF-κB-regulated genes are anti-apoptotic and include the protein families of Bcl-2 and inhibitors of apoptosis (IAP). IAP suppresses apoptosis by direct inhibition of

proteolytic caspases. X-chromosome linked IAP (XIAP) is the most potent inhibitor of apoptosis but its regulation by NF-κB seems to be limited to specific cell types (176). Although IAP-1 and IAP-2 have reduced ability to inhibit apoptosis compared to XIAP, their induction in response to activation of NF-κB has been observed in various cells (176, 177). It is well known that activation of NF-κB induces transcriptional activation of Bcl-xL in many different types of cells. Recent studies also suggested the involvement of Bcl-2 in the anti-apoptotic activity of NF-κB (175, 176). It is well understood that high expression of Bcl-xL and Bcl-2 inhibits apoptosis through the suppression of the release of cytochrome *c* from mitochondria (178, 179).

On the other hand, p53 activity seems to be inhibited in the AOM-treated colon. Shortly after the AOM injection to mice, p53-regulated genes were not induced in the colon in contrast to rapid induction of p53-responsive genes including p21 and Bax in the liver (169). In addition, Wu et al. (23) reported that cytosolic wild type (wt) p53, which is stable and inactive, was increased whereas active mitochondrial wt p53 decreased in the colon after AOM treatment although levels of wt p53 mRNA and total proteins were not inhibited by AOM. Therefore, activation of NF-κB may have an important role in modulating cell death in the AOM-treated colon.

AOM induces apoptosis in the colonic epithelium within several hours after AOM injection in young animals (166). However, the time course of apoptosis upon exposure to AOM in older animals has not been studied. This study investigated the effect of aging on the regulation of apoptosis in response to an acute exposure to AOM. In this study, the time course of apoptotic response in different ages of animals was investigated to determine whether older animals respond excessively or bluntly to the exposure of colonic cytotoxicants compared to young animals. Also, NF-κB-regulated genes, Bcl-2, Bcl-xL, and IAP-2 were examined to assess whether differences in apoptosis induction by age involves regulation of those genes.

5.2 Materials and Methods

5.2.1 Experimental animals and diets

Experimental animals were young (6 weeks), middle-aged (12 months), and old (22 months) male F344 rats purchased from the colony at the National Institute of Aging (Bethesda, MD). The rats were fed the AIN-93 diet for 1 week after acclimation to lab conditions. All the rats except 6 rats per age group were given single s.c. injection of AOM (Sigma, St. Louis, MO) at the dose of 15 mg/kg. Six rats per age group were killed at four different time points, 4, 8, 16, and 24 hours after

AOM injection. The zero hour control was injected with saline right before they were killed as a sham control. Colons were collected after flushing with cold saline. Two cm of colon tissue from the distal end were fixed in 10% formaldehyde for immunohistochemistry and the rest of the colon was scraped to collect epithelial mucosa. One half of the mucosa from each rat was fixed in RNAlater (Ambion Inc, Texas, AU) for RNA isolation and the other half was frozen on liquid N_2 for protein analysis. Tissues were then frozen at –80 ^{o}C until analyzed.

5.2.2 Apoptosis

Paraffin-embedded colon pieces were subjected to immunohistochemistry using ApopTag Peroxidase kits (ApopTag 700, Chemicon International, Inc., Temecula, CA) according to the manufacture's instructions, and apoptotic indices were determined by counting the total number of stained cells per crypt. Briefly, DNA 3'-OH ends were labeled with oligonucleotides containing digoxigenin conjugate by terminal deoxynucleotidyl transferase. Labeled cells were detected by anti-digoxigene conjugated with peroxidase reporter and subsequent reaction with diaminobenzidine (DAB) substrate.

5.2.3 Transcriptional levels of Bcl-2, Bcl-xL, and IAP-2 using RT-PCR

Total RNA was extracted from colon mucosa and reverse transcribed to cDNA as described (Section 2.2.3). An aliquot of cDNA was subjected to RT-PCR for each gene using specific oligonucleotides for Bcl-2 (180), Bcl-xL (181), and IAP-2 (sense: AAA TGC TGA CCC TCC AG, antisense: AAA TGC TGA CCC TCC ACT TG, Invitrogen). The DNA engine (MJ Research, Waltham, MA) was used for mRNA amplification of each gene. Thermocycling conditions were used as follows: one 2-min cycle at 94 ^{o}C, 30 (IAP-2 and Bcl-Xl) or 32 (Bcl-2) cycles of denaturation for 30 s at 92 ^{o}C , annealing for 30 s at 57 ^{o}C (Bcl-xL) or 60 ^{o}C (IAP-2 and Bcl-2), and extension for 1 min at 72 ^{o}C, and a final extension for 5 min at 72 ^{o}C. Ribosomal 18S was used as an internal control and the PCR products from multiplex reactions were analyzed using DNA 500 LabChip® and Agilent 2100 bioanalyzer as previously described (Section 2.2.3).

5.2.4 Data analysis

This study was a two factorial (age × time) design. Two-way analysis of variance (ANOVA) was conducted using SAS software (9.1, SAS Institute Inc, Cary, NC) to investigate the effect of age, time and their interaction on apoptosis and transcriptional levels of each gene.

5.3 Results

5.3.1 Apoptosis

An apoptotic peak in the colon was reached 8 hours after AOM injection and after that apoptotic incidence decreased with time but was still elevated 24 hours after the carcinogen treatment compared to basal levels in all age groups (Fig. 14). Older animals had significantly higher apoptotic indices (total stained cells/crypt) at 8 ($p < 0.05$) and 16 hours ($p < 0.07$) after AOM injection, compared to young animals. When two-way ANOVA was conducted, there were significant differences in effects of age, time, and the interaction of age and time.

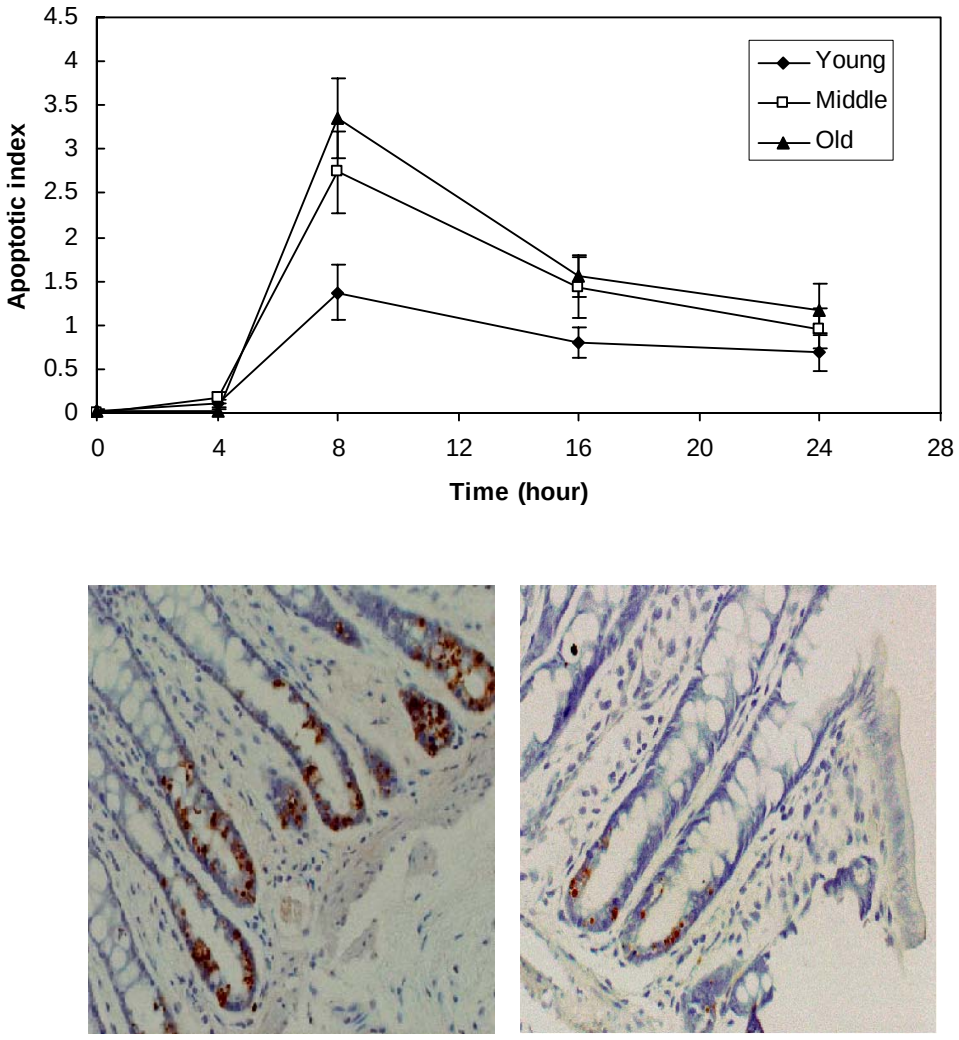

Figure 14. Time course of apoptosis indices in the distal colon of young, middle aged and old rats after being treated with AOM (mean ± SE, n=5 or 6/group). Apoptotic index was generated by counting total stained cells per crypt. Except for 24 hour point in young rats, apoptotic indices were higher ($p < 0.05$) at 8, 16, and 24 hour points compared to 0 or 4 hour point in each age group. Middle-aged and old rats (lower panel, left) had significantly higher ($p < 0.05$) apoptotic indices compared to young rats (lower panel, right) at 8 hour time point. Two-way ANOVA indicated significant effects of age, time, and interaction of age and time.

5.3.2 Transcriptional levels of Bcl-2, Bcl-xL, and IAP-2

Changes in colonic transcriptional levels of Bcl-2, Bcl-xL and IAP-2 with time after AOM injections showed a similar trend in each age group – mRNA levels of genes were lowered after carcinogen treatment in young and old rats whereas in middle-aged rats, they were unchanged following AOM injections (Fig. 15). Two-way ANOVA showed that there was a significant interaction of age and time in Bcl-xL and Bcl-2 mRNA levels.

Young rats showed the most apparent change of mRNA expression levels of the genes after AOM treatment. In young rats, transcriptional levels of all of the genes, Bcl-xL, IAP-2, and Bcl-2, were significantly reduced ($p < 0.05$) 24 hours after the AOM treatment compared to 0 hour control (Fig. 15). In old group, Bcl-xL and IAP-2, but not Bcl-2 mRNA levels, were significantly decreased following AOM treatment. There were no significant changes in transcriptional levels of Bcl-xL, IAP-2, or Bcl-2 after AOM injections in middle-aged rats.

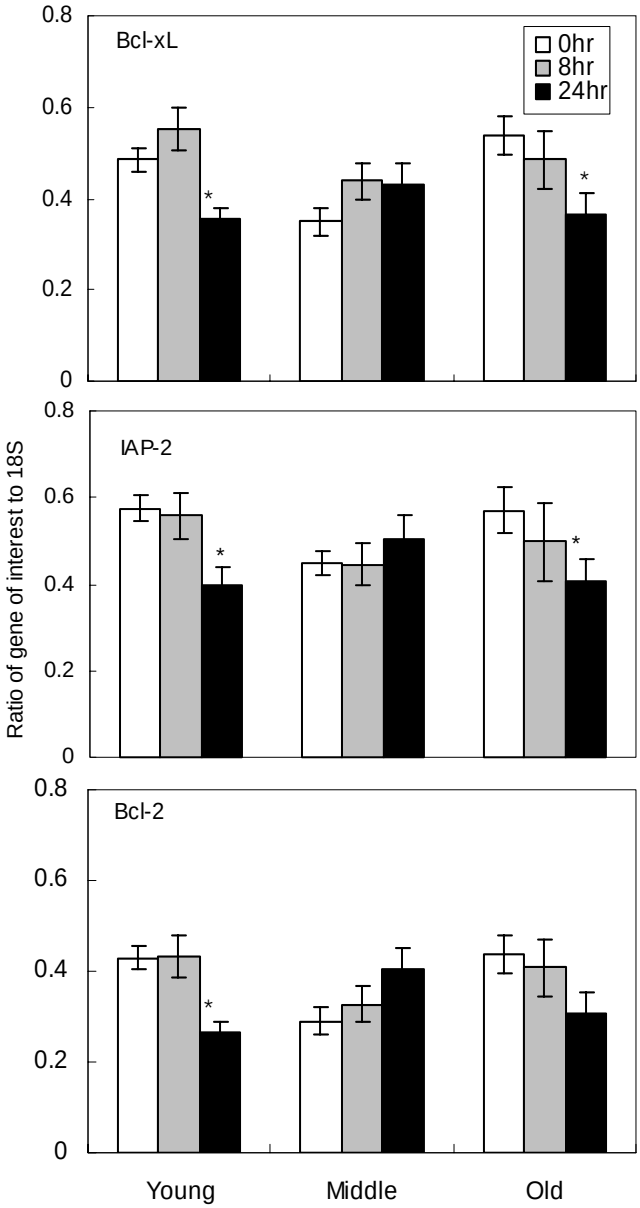

Figure 15. Changes in transcriptional levels of Bcl-xL, IAP-2, and Bcl-2 in relation to 18S in the colon of three different ages of rats 0, 8 or 24 hours after AOM injection (mean ± SE, n=5 or 6/group). * represents significant differences ($p < 0.05$) compared to 0 hour control in each age group by ANOVA.

5.4 Discussion

Apoptosis is a critical mechanism in maintaining tissue homeostasis. Two apoptotic pathways are known. One is the external pathway where death-receptors are involved and the other is the mitochondrial-dependent pathway, called the internal pathway. Cellular apoptotic events are highly regulated by many proteins whose expressions are controlled by many different signaling pathways as described above (Section 1.6). The regulation of apoptosis varies by stimuli and intensity of apoptosis, and depends on cell types. Even more complicated, the two apoptotic pathways interact and induction of one apoptotic pathway requires amplification of apoptotic signaling by the other pathway (100).

Deregulation of apoptosis seems to contribute to the aging phenotype. In post-mitotic tissues, apoptosis increases with age and causes loss of tissue structure and function due to loss of cells as seen in muscle atrophy, sarcopenia, and neurodegenerative diseases (174, 182, 183). However, it is largely unknown how aging contributes to the regulation of apoptosis in mitotic tissues including the colon. The current study confirmed previous results that basal levels of apoptosis were similar among different age groups (109) and that maximum levels of apoptosis were reached 8 hours after the AOM injection in young rats (166). Aging did not alter the time at which apoptosis reaches a peak (Fig. 14). However, the levels of AOM-

induced apoptosis at the peak were higher in older animals compared to young. Therefore, this study showed that apoptosis increases with age upon exposure to a genotoxic agent in the mitotic tissue, the colon, as demonstrated in many post-mitotic tissues.

Upon exposure to DNA damaging agents, apoptosis functions to remove damaged DNA and reduce mutation load in tissues (184, 185). Therefore, apoptosis is a critical mechanism to prevent initiation of cancer. In the first study (Chapter 2), the development of ACF after AOM treatment was lower in middle-aged and old compared to young rats. Higher incidence of apoptosis immediately after the AOM treatment in older animals may inhibit the initiation of colon cancer and therefore explain the reduced development of colonic preneoplastic lesions, ACF.

On the other hand, high incidence of apoptosis may eventually cause loss of functional cells in the colon. Apoptosis depletes the stem cell pools that replenish renewable tissues (186, 187). Notably, the majority of AOM-induced programmed cell death occurred in the bottom of crypts in both young and old animals (Fig. 14). The fact that AOM-induced apoptosis targets stem cells was also previously observed (154). By contrast, in the normal colon, apoptotic cells are often found in the upper portions of the crypts where highly differentiated cells reside and are exfoliated into the lumen for excretion (154). How this high incidence of apoptosis contributes to

the aging phenotype in the colon needs further study. However, it might be reasonable to assume that a high incidence of apoptosis in the colon of older rats may potentially contribute to loss of structure and function due to loss of functional cells as demonstrated in post-mitotic tissues. It should be also noted that destruction of tissue structure can deregulate cell proliferation, differentiation and apoptosis (188-190) and that modification of tissue structure can actually initiate cancer (191).

Another possible mechanism by which over-activation of apoptosis may increase the cancer development is the stimulation of cell proliferation to compensate for loss of cells, thereby, potentially providing more chances to establish the mutations by replication. In fact, basal levels of cell proliferation determined by proliferating cell nuclear antigen (PCNA) were slightly but significantly higher in older rats compared to young rats (Chapter 6). A previous study (166) showed that three days after AOM single injection, cell proliferation rate was significantly elevated compared to basal levels in young rats, probably in order to replenish lost cells. Cell proliferation rate was significantly reduced 16 hours after AOM injection compared to basal levels only in old rats whereas proliferation rate was not altered in either young or middle-aged rats (Chapter 6). If loss of cells due to apoptosis increases cell proliferation even higher than basal levels in old rats as in young rats (166), cell proliferation would dramatically rise in old animals probably much higher

than young. A high incidence of cell proliferation is considered to increase risk of cancer development due to the increased chance of a mutation leading to preneoplastic lesions. Repeated exposures to genotoxic agents may have substantial impact on individuals highly susceptible to apoptotic stimuli. In the short term, high incidence of apoptosis in response to AOM in older animals has a beneficial effect in prevention of initiation of colon cancer. In the long term, however, the high susceptibility to induced apoptosis may potentially contribute to the aged phenotype and increased cancer incidence due to loss of tissue structure and deregulation of cell proliferation, providing an environment more conducive to cancer initiation and promotion.

In this study, changes in the expression of NF-κB-regulated genes following AOM treatment were examined. Bcl-2, Bcl-xL, and IAP-2 transcriptional levels were all similarly affected by AOM treatment in three different aged rats. Interestingly, except for Bcl-2, these three genes were transcriptionally less expressed 24 hours after AOM injection compared to 0 hour control in young and old animals (Fig. 15). On the contrary, in middle-aged rats, none of these genes were significantly reduced after AOM injection. The protein products of these genes inhibit apoptosis. Assuming that the change in messages of Bcl-2, Bcl-xL, and IAP-2 are translated to protein levels, inhibitors of apoptosis would be decreased in the

colon of young and old rats and increase sensitivity to apoptosis, in contrast to no changes in the colon of middle-aged rats. The change in transcriptional expression levels of those genes did not appear to relate with age-related differences in apoptosis induction, as if this were the case, the lowest levels of apoptosis should have been observed in middle-aged rats.

Bcl-2, Bcl-xL, and IAP-2 proteins seem to play critical roles in inhibiting the mitochondrial-dependent pathway. IAP family inhibits caspase-9 and hence the cleavage of pro-caspase-3 to the active form whereas no apparent effect of IAPs was reported in activation of caspase-3 by caspase-8 (192, 193). Bcl-2 and Bcl-xL inhibit cytochrome *c* release from mitochondria and therefore suppress apoptosis mediated by the mitochondrial-dependent pathway (194). Whether high expression of Bcl-2 or Bcl-xL also has an inhibitory effect on death receptor-mediated pathway of apoptosis is controversial (100). This seems to depend on the type of cells (100). A certain type of cells is resistant to apoptosis induced by death ligands. This type of cells has low death receptor levels, is resistant to cross linking of receptors, or possibly expresses inhibitory decoy receptors. Also, this type of cells has high levels of anti-apoptotic proteins like IAPs and require the release of mitochondrial Smac/DIABLO, which sequester IAPs in order to activate caspase-9 and caspase-3. This release of Smac/DIABLO from mitochondria is inhibited by Bcl-2 family.

Therefore, in this type of cells, Bcl-2 or Bcl-xL has inhibitory effect on death receptor-mediated apoptosis as well. It should be confirmed that transcriptional levels of Bcl-2, Bcl-xL and IAP-2 were translated to protein levels. The mitochondrial-independent pathway, which is not affected by IAPs or Bcl-2/Bcl-xL, might be responsible for the age-related differences in AOM-induced apoptosis.

Many chemicals that induce DNA damage are known to induce apoptosis through the mitochondrial-dependent pathway or both internal and external pathways. It is not well understood how AOM induces apoptosis in the colon. Several studies also reported that production of cytokines, including TNF-α and IL-6, is elevated in AOM-treated colon (170, 195, 196) although it is not clear whether levels of cytokines are increased directly by AOM treatment or during progression of carcinogenesis. Interestingly, it is well established that production of TNF-α and IL-6 increases in older individuals (197). Moreover, old subjects not only produce higher TNF-α but also are more susceptible to TNF-α-induced apoptosis compared to younger individuals (197). It would be interesting to investigate if older rats increase colonic production of cytokines including TNF-α or IL-6 in response to AOM exposure or are more sensitive to apoptosis induced by those cytokines.

In summary, this study showed that the colons of older animals are more susceptible to AOM-induced apoptosis and this age-related difference in the incidence

of apoptosis is likely not related with NF-κB activity. Further study will be required to identify the apoptotic pathway that AOM induces and to understand the biological significance of high susceptibility of older animals to apoptosis upon an exposure to a colon carcinogen.

Chapter 6. Effect of aging on colonic apoptosis induced by AOM and curcumin

6.1 Introduction

Middle-aged rats are resistant to inhibition of colonic ACF development by dietary curcumin (Chapter 2). ACF represent a stage of initiation and early promotion in colon carcinogenesis. In the early stages of carcinogenesis, the dynamics of carcinogen metabolism, repair of DNA mutation, cell proliferation and apoptosis determine the mutation load in the tissue and the initiation of carcinogenesis. The induction of apoptosis may be an important mechanism in the inhibition of AOM-initiated colon carcinogenesis. Shortly after the AOM injection, p53-regulated genes were not significantly induced in the colon of mice whereas they were markedly enhanced in the liver (169), indicating suppression of apoptosis or cell cycle arrest by AOM treatment in the colon. Also, the efficacy of chemoprevention appears to be related with the ability of chemopreventive agents to induce apoptosis. Samaha et al. (198) reported that the high tumor incidence (number of rats bearing tumors) was correlated with a lower apoptotic index when they used different chemopreventive agents. Moreover, studies that investigated the chemopreventive activity of pectin and fish oil in AOM-induced colon cancer suggest that the chemopreventive effect of

diet may be due to the increase of apoptosis not the decrease of cell proliferation at all stages of carcinogenesis, initiation, promotion, and progression (33, 153, 199).

The induction of apoptosis by curcumin and potential apoptotic pathways that curcumin may mediate has been extensively studied (84, 95, 96, 104, 105, 200, 201). Most were conducted *in vitro* and the apoptotic pathways affected by curcumin varied depending on cell line types and concentrations of curcumin used. However, many studies suggest that curcumin induces apoptosis through the mitochondrial-dependent pathway. In addition, a recent study (202) showed that young rats fed curcumin had higher active caspase-9 expression levels, indicating that curcumin-induced apoptosis is mediated by the mitochondrial-dependent pathway.

Based on previous studies above, the resistance of middle-aged rats to curcumin chemoprevention (Chapter 2) may be due to the failure of curcumin to enhance apoptosis in middle-aged rats. One of the potential mediators may be Hsp70 in the colon as described previously (Section 4.1). However, the preliminary study (Chapter 4) investigating the time course of Hsp70 mRNA expression after AOM injection did not provide conclusive results. It would be relevant to first determine whether age affects curcumin-induced apoptosis rather than search for responsible modulators. Regulation of apoptosis is a significant event in both the aging process and cancer prevention as reviewed above. Moreover, the study

described in Chapter 5 showed significant age-related differences in AOM-induced apoptosis.

Therefore, this study investigated whether age affects curcumin-induced apoptosis and whether it is related to the resistance to chemopreventive activity of curcumin in middle-aged animals. In this study, the time course of Hsp70 expression following AOM injection was also examined to determine if AOM induces Hsp70 mRNA expression, and if the induction of Hsp70 has a regulatory role in curcumin-induced apoptosis. Additionally, the activity of caspase-9 was assessed to investigate if curcumin-induced apoptosis is mediated through the mitochondrial-dependent pathway.

6.2 Materials and Methods

6.2.1 Experimental animals and diets

Male F344 rats at the ages of 6 weeks, 12 months, and 22 months were purchased from the same colony described above (Section 5.2.1). Rats were randomly allocated into either AIN or CUR diet groups. All the rats were injected with AOM at the dose of 15 mg/kg body weight after 1 week on their diets. Eight and sixteen hours after the AOM single injection, rats were killed. Rats in the 0 hour

control group were injected with saline right before they were killed. These rats represented basal levels. A 2 cm colon piece from the mid point towards the distal colon, and one from the distal end were fixed in 10% formaldehyde for immunohistochemisty and the rest of colon was scraped for mucosa and stored for RNA and protein isolation as described (Section 5.2.1).

6.2.2 Apoptosis

Incidence of apoptosis was assessed by immunohistochemical staining using ApopTag Peroxidase Kits (Chemicon International, Inc., Temecula, CA) and apoptotic indices of total stained cells per crypt were generated as described (Section 5.2.2). Two sections (one from distal and the other from middle of the colon) of each colon were used for apoptosis to determine if results differed with the region of the colon.

6.2.3 Cell proliferation

Paraffin-embedded colons from two locations (one from distal and the other from middle of the colon) were sectioned and subjected to immunohistochemical staining for PCNA. Tissue sections were incubated with primary antibodies against PCNA (predilute, Zymed Laboratories, South San Francisco, CA) for one hour at room temperature. Histostatin-Plus kits (DAB, Broad Spectrum, Zymed) were used

to successively conjugate primary antibodies with biotinylated secondary antibodies, streptavidin-peroxidase conjugates and diaminobenzidine (DAB) substrates.

Computer-Assisted Image Analysis was employed to quantitatively measure immunohistochemically stained PCNA. Each stained slide was viewed at 100X with a Nikon Eclipse microscope mounted with a high-resolution camera. Images were obtained from two locations of each section and a mean labeling index was calculated as the percentage of the positive nuclear-stained area in the total nuclear area using a Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Silver Spring, MD). Only complete crypts with a clear opening to the lumen were selected for analysis.

6.2.4 Transcriptional levels of Hsp70 by RT-PCR

Hsp70 mRNA levels were measured by RT-PCR and the ratio of Hsp70 to 18S was determined as previously described (Section 4.2.2).

6.2.5 Caspase-9 protein levels by Western blotting

Frozen colon mucosa samples were lysed with T-PER tissue protein extraction reagent (Pierce Biotechnology, Inc., Rockford, IL) containing protease inhibitors (Halt protease inhibitor cocktail, Pierce Biotechnology). Tissue homogenates were centrifuged at 10,000 g for 5 min at 4 ^{o}C. Aliquots of tissue

supernatant were mixed with sample loading buffer (50 Mm Tris buffer pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.02% bromophenol blue) and denatured at 95 ^{o}C for 5 min. Denatured proteins were loaded onto 10-20% gradient Novex® Tris-Glycine gels (Invitrogen, Carlsbad, CA). After electrophoresis, proteins were transferred onto nitrocellulose membranes and probed with caspase-9 antibody (MBL International, Woburn, MA). The Western blot was visualized using Super Signal West Pico Chemiluminescent Substrate (Pierce Biotechnology). The densitometry of the bands corresponding to the molecular size of active caspase-9 (35 kD) and procaspase-9 (45 Kd) were quantified using Image J software (1.35g, NIH) and the ratio of active caspase-9 to procapase-9 was generated for each rat. There was no difference among samples in intensity of bands of β-actin used as a loading control. Protein concentration of tissue lysates was measured using Coomassie Plus protein assay kit (Pierce Biotechnology).

6.2.6 Data analysis

Two-way analysis of variance (ANOVA) using SAS software (9.1, SAS Institute Inc, Cary, NC) was performed to examine the effect of diet, time, and their interaction on the ratio of Hsp70 to 18S mRNA levels and the ratio of active caspase-9 to procaspase-9 in each age group. Two-way ANOVA was also conducted to

determine if there were significant effects of age, diet, and their interaction on apoptotic index in each time after the AOM injection due to high variability of values depending on the time at which apoptosis was evaluated.

6.3 Results

6.3.1 Apoptosis

There was a significant interaction effect ($p < 0.05$) of age and diet at all time points. Curcumin-fed young and old rats showed significantly higher basal levels (0 hour) of apoptosis in the colon in contrast to no effect of curcumin in middle-aged rats (Fig. 16). Dietary curcumin also enhanced AOM-induced levels of apoptosis in young and old rats, at 8 and 16 hours, respectively, compared to their control diet counterparts whereas middle-aged rats had similar levels of apoptosis in both diet groups in all time points (Fig. 16). Results from the middle colon were similar, but less apparent as in distal colon (data not shown).

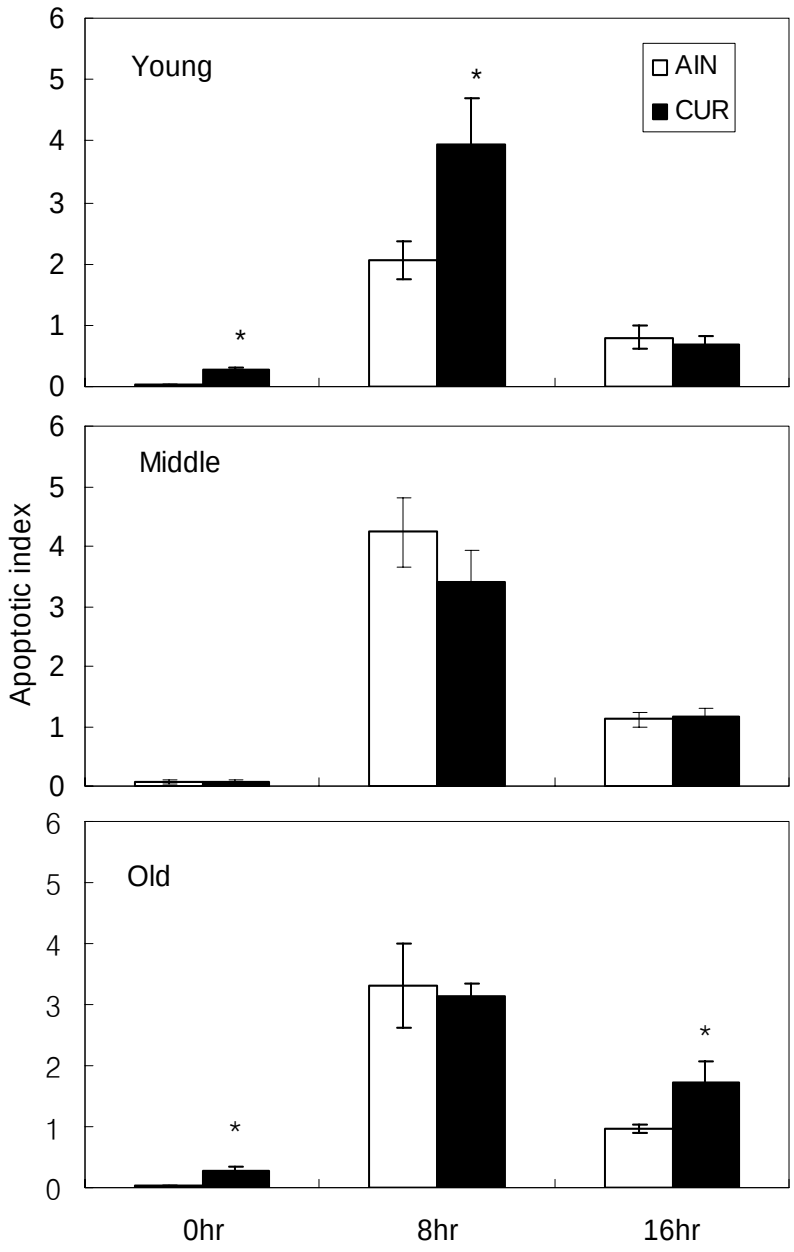

Figure 16. Effect of curcumin on levels of basal and AOM-induced apoptosis in the distal colon of three different aged rats (mean ± SE, n=5 or 6/group). AIN is the group fed the AIN-93 control diet and CUR is the group fed the AIN-93 diet containing 0.6% curcumin. Apoptosis was detected by immunohistochemical staining 0, 8, and 16 hours after AOM injection. Apoptotic indices were generated by counting total number of stained cells per crypt. * represents significant differences ($p < 0.05$) between AIN and CUR at each time point and each age group by ANOVA.

6.3.2 Cell proliferation

Changes of cell proliferation rate in distal colon expressed as PCNA labeling index is shown in Table 3. In control diet group, basal levels (0 hour) of PCNA labeling indices were significantly higher ($p < 0.05$) in middle-aged and old rats compared to young rats in the distal colon. Feeding curcumin did not significantly affect basal levels of cell proliferation in any ages of rats. Carcinogen treatment significantly reduced ($p < 0.05$) the proliferation rate in all ages of rats fed curcumin. In the control diet group, only old rats had a significantly reduced cell proliferation rate following AOM injection, but this was still higher than in rats fed the curcumin diet. Therefore, after carcinogen treatment, rats fed the curcumin diet had significantly lower ($p < 0.05$) levels of PCNA staining compared to their control diet counterparts in all three ages. There were no significant changes by age, diet, or time after AOM treatment in the middle colon (data not shown).

Table 3. PCNA labeling index (%) in distal colons of three different aged rats before and after AOM injection

Time[a)]	Age / Diet[b)]	Young	Middle-aged	Old
0 hour	AIN	13.6 ± 1.79[c)]	22.6 ± 3.00[d)]	28.8 ± 3.93[d)]
	CUR	12.8 ± 3.31	17.1 ± 3.23	20.5 ± 4.09
16 hour	AIN	13.3 ± 1.86	15.4 ± 1.64	14.7 ± 3.22[f)]
	CUR	6.9 ± 1.59[e)]	5.0 ± 0.82[e),f)]	6.2 ± 1.43[e),f)]

[a)] Rats were killed 0 or 16 hours after AOM injection

[b)] AIN is AIN-93 control diet and CUR is AIN-93 diet containing 0.6% curcumin

[c)] PCNA labeling index was calculated by percentage of nuclear-stained area of the total nuclear area, mean ± SE (n=5 or 6 per group). Data values were log transformed due to large difference in variance among different groups for ANOVA.

[d)]Significant difference ($p < 0.05$) among different age groups at each time point and each diet group by ANOVA

[e)]Significant difference ($p < 0.05$) between diet groups at each time point and each age group by ANOVA

[f)]Significant difference ($p < 0.05$) between time points at each age and each diet group by ANOVA

6.3.3 Hsp70 mRNA levels

In young rats, Hsp70 mRNA levels were increased after AOM injections in the control diet group. Young rats fed the control diet had significantly higher ($p < 0.05$) Hsp70 levels 16 hours after AOM treatment compared to basal levels (Fig. 17). This elevated level of Hsp70 mRNA in young rats was significantly lowered in rats fed curcumin (Fig. 17). However, there were no significant differences ($p > 0.05$) by either AOM treatment or diet in old animals (Fig. 17) and results similar to old were observed in middle-aged animals (data not shown).

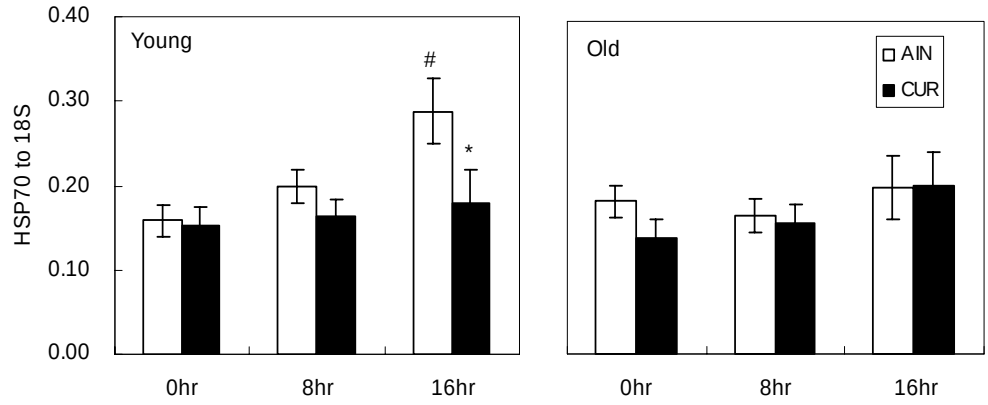

Figure 17. Changes in Hsp70 transcriptional levels 0, 8, and 16 hours after AOM injection in the colon of young and old rats fed either AIN or CUR (mean ± SE, n= 5 or 6/group). AIN is the group fed the AIN-93 control diet and CUR is the group fed the AIN-93 diet containing 0.6% curcumin. * represents significant differences ($p < 0.05$) between AIN and CUR at each time point and each age group. $^{\#}$ represents significant differences ($p < 0.05$) among different time points at each age and each diet group by ANOVA. There were no significant effects of diet or AOM treatment in old animals. Results similar to old were observed in middle-aged animals (data not shown).

6.3.4 Caspase-9 protein levels

As shown in Figure 18, in the young, rats fed the curcumin diet had a higher ratio of active caspase-9 to procaspase-9 compared to rats fed the control diet in all times consequently showing a significant diet effect ($p = 0.004$). The ratios of active caspase-9 to procaspase-9, which reflect the activity of caspase-9 were significantly increased 0 and 8 hours after AOM injection in young rats fed curcumin diet compared to rats fed the control diet (Fig. 18). There were no significant differences between two diet groups in all time points in middle-aged or old rats (Fig. 18).

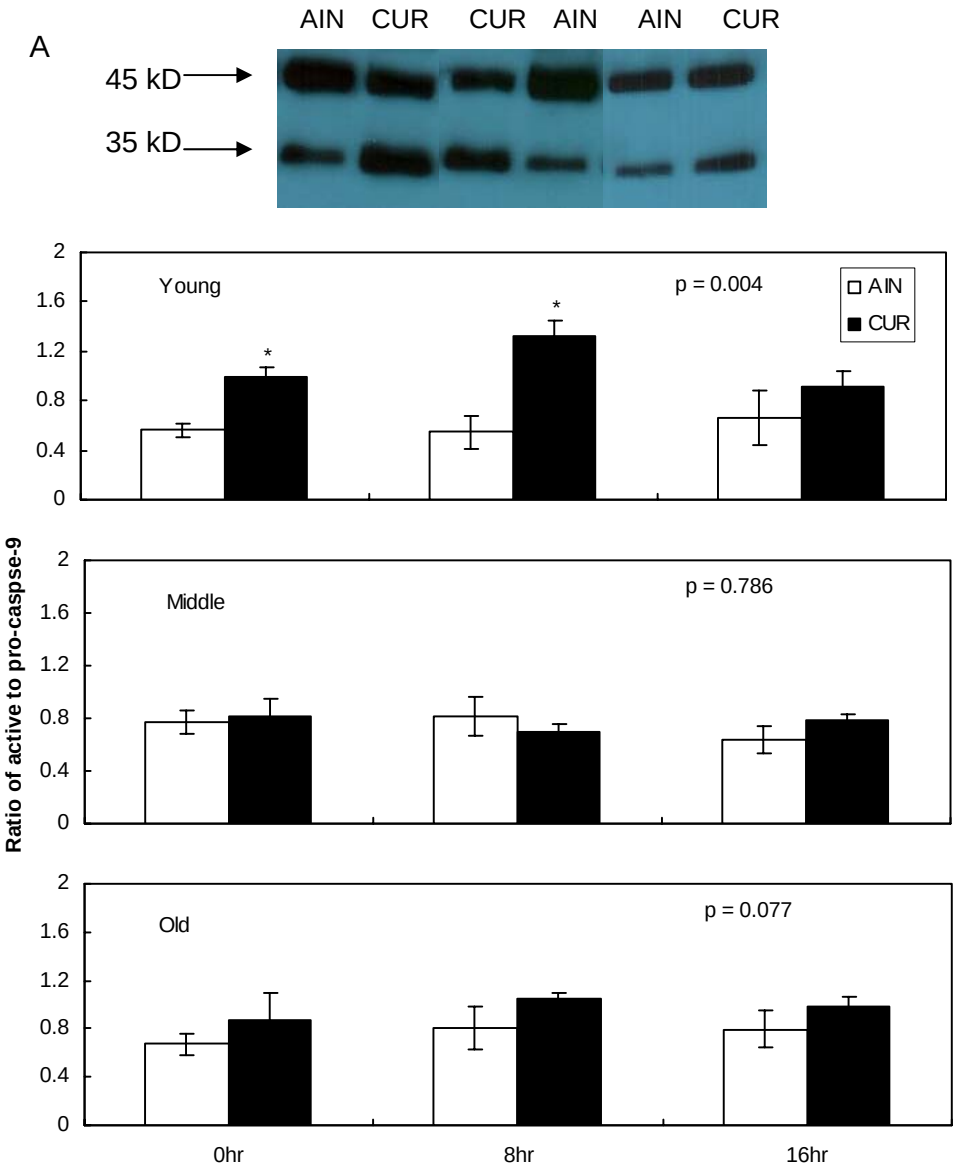

Figure 18. Effect of curcumin on the activity of caspase-9 in the colon of three different ages of rats, 0, 8, and 16 hours after AOM injection (mean ± SE, n=5 or 6/group). (A) Proteins were separated by electrophoresis and blots were probed with caspase-9 antibody, lane 1-2; 0 hour, lane 3-4; 8 hour, lane 5-6; 16 hour in young rats. Bands of 45 kD and 35 kD corresponded to procaspase-9 and active caspase-9, respectively. (B) The ratio of active caspase-9 to procaspase-9 was calculated after the densitometry corresponding to the size of two proteins was quantified by Image J software. β-actin was used as a loading control and there was no difference in intensity of β-actin protein among samples (not shown). p values represent the overall effect of diet in each age group. * represents significant differences ($p < 0.05$) between AIN and CUR at each age group and each time point by ANOVA.

6.4 Discussion

The hypothesis of this study was that the resistance of middle-aged rats to curcumin-induced apoptosis at the initiation of colon carcinogenesis contributes to the failure of curcumin to inhibit colonic ACF in this age group. The results support this hypothesis as young and old rats fed the curcumin diet had higher levels of apoptosis compared to rats fed the control diet whereas middle-aged rats had similar levels of apoptosis in both diet groups. Curcumin similarly affected cell proliferation assessed by PCNA immunohistochemical staining in all age groups and significantly reduced proliferation rate after AOM injection (Table 3). This result support previous reports that the ability to enhance apoptosis or differentiation rather than suppress cell proliferation correlates with efficacy of chemopreventive agents (33, 153, 199). Therefore, induction of apoptosis by curcumin before and/or right after carcinogen treatment may be at least partially responsible for the differential chemopreventive activity of curcumin (Chapter 2).

Apoptosis may be important in inhibition of not only initiation of cancer but also progression of initiated cancer (33, 198). Volate et al. (202) recently reported that curcumin reduced the number of colonic ACF induced by AOM in young rats and that this chemopreventive activity of curcumin was related with the increase of apoptotic incidence. This is in agreement with the finding in this study as young and

old rats fed curcumin had significantly higher basal levels of apoptosis. At this time, no reports were found to explain what contributes to the higher apoptosis incidence by curcumin-fed untreated rats or what are the biological consequences of this observation. However, higher incidence of apoptosis in the AOM-untreated colon may provide protection against endogenous carcinogens in the colon, which is continuously exposed to toxic substances. Therefore, through induction of apoptosis, curcumin may suppress the initiation of cancer, the development of initiated cancer cells to preneoplastic lesions and/or the growth of ACF with small multiplicity to ACF with larger multiplicity.

As described earlier (Section 1.6), activation of procaspase-9 is mediated by the mitochondria. The increase of apoptosis in rats fed curcumin was significantly associated with higher active caspase-9 relative to procaspase-9 in young rats (Fig. 18). However, this relationship was not observed in old animals, suggesting potential involvement of other pathways. It should be also noted that the ratio of active- to pro-caspase-9 did not change with time after AOM injection in all age groups. AOM-induced apoptosis may not require activation of caspase-9 but may be mediated by a mitochondrial-independent pathway.

The mechanism of resistance to curcumin-induced apoptosis in middle-aged rats is unclear. Previously, it was reported that curcumin failed to induce apoptosis

in cells which highly express Hsp70 (203) or Bcl-2 (85). In this study, changes in transcriptional levels of Hsp70 with time after AOM treatment were measured to find if Hsp70 expression modulates curcumin-induced apoptosis. Hsp70 was transcriptionally induced 8 hours after AOM treatment in young rats, reaching levels statistically significant at 16 hours. This result suggests that the failure in detection of induction of Hsp70 transcription following AOM treatment in the previous study (Chapter 4) may be because Hsp70 transcription was evaluated at a much later time point after AOM treatment. However, the feeding of curcumin in young rats reduced the elevation of Hsp70 mRNA levels. Therefore, curcumin may act to eliminate stress rather than enhance Hsp70 induction as initially hypothesized in Chapter 4. Methylated proteins may induce transcriptional activation of Hsp70 in the colon following AOM treatment. Young rats fed curcumin showed increased apoptosis incidence after AOM treatment compared to rats fed control diet and this removal of damaged cells by apoptosis may result in the reduction of protein damage that would induce Hsp70 in the colon. In middle and old rats, Hsp70 does not appear to be induced by AOM and transcriptional levels of Hsp70 were not affected by feeding of curcumin either. The lack of induction of Hsp70 in older rats may be because the higher incidence of apoptosis after AOM treatment in middle and old compared to young animals effectively removed stressed cells or because older animals have

reduced ability to induce Hsp70 (161, 204).

The study above investigating time course of apoptosis in three different ages of rats (Chapter 5), showed no changes in Bcl-2, Bcl-xL, and IAP-2 mRNA levels in the colon of middle-aged rats after AOM treatment. This is in contrast to the decrease of expression of those genes in young and old rats. Bcl-2, Bcl-xL, and IAP-2 are regulated by activity of NF-κB and responsible for the resistance to apoptosis induction (176). Although protein levels of those genes should be confirmed and the apoptotic pathway curcumin induces identified, the lower expression of Bcl-2, Bcl-xL, and IAP-2 suggests a means of sensitizes young and old rats to curcumin-induced apoptosis.

Apoptosis mainly occurred at the bottom of the colon crypts in rats treated with AOM in both the previous (Chapter 5) and the current study. However, rats fed the curcumin diet had more apoptosis in the middle or upper areas of crypts compared to rats fed the control diet after AOM treatment. The differential localization of apoptosis by diet may be due to the enhanced differentiation and migration of cells in the curcumin diet group as suggested by Fenton et al. (205). Cell differentiation is an important characteristic that indicates maintenance of tissue function. It should be also noted that aging seems to increase apoptosis in post-mitotic tissues and probably mitotic tissues as well, as demonstrated in our previous study (Chapter 5).

Therefore, increasing apoptosis without enhancing cell differentiation would not be desirable as it would cause loss of tissue structure and function, further contributing to aging. It should be further confirmed if curcumin enhances cell differentiation. In this study, apoptotic incidence was enhanced by curcumin at a later time point after AOM treatment in old compared to young rats (Fig. 16). The mechanism of this delay of increase of apoptosis by curcumin in old rats is also unclear.

A previous study (109) reported that basal levels of cell proliferation determined by bromodeoxyuridine (BrdU) incorporation were not different among different ages of male F344 rats in contrast to increased proliferation in the colonic epithelium in older rats in this study (Table 3). Hirose et al. (166) reported that PCNA labeling index was not reduced 8 hours after AOM injection when actively proliferating cells in the colonic epithelium are expected to be rare as mitotic index was significantly suppressed and apoptosis was highly induced at this time point. They speculated that cells in not only S phase but also G1 and/or G2 phase may immunoreact for PCNA. PCNA plays important role both in DNA synthesis and DNA excision repair. Possible occurrence of DNA excision repair after AOM treatment was also suggested as another explanation of no suppression of DNA synthesis assessed by PCNA labeling index after AOM treatment (166). Therefore, different techniques used to assess cell proliferation might give different results.

Another possibility was that cell proliferation may be evaluated in tissues from different locations of the colon. Holt and Yeh (206) reported an increased proliferation rate and a wider proliferation zone in aged rats in the distal colon but no changes were found in the proximal colon when they employed the tritiated thymidine incorporation techniques to assess cell proliferation rate. In present study, there were no differences in cell proliferation by age, AOM treatment, or diet in the middle colon. A previous study also suggested that differential sensitivity to AOM depending on location in the colon (206) . AOM treatment causes damages predominantly to the distal colon and changes of cell proliferation and mitosis were more apparent in the distal colon following AOM single injection (206) . Therefore, the distal colon might be more susceptible to carcinogens, age, and probably diet.

In summary, this study supports the hypothesis that the resistance of curcumin-induced apoptosis in middle-aged rats during initiation of AOM-induced carcinogenesis contributes to the lack of inhibition of colonic ACF by curcumin in this age of animals. Further study will be required to identify the apoptotic pathways and mediators contributing the resistance to curcumin-induced apoptosis in middle-aged animals.

Chapter 7. Conclusions and Future Study

Aging is a potent factor driving cancer including colorectal cancer. The incidence of colorectal cancer, one of leading neoplastic diseases in Western countries, is strongly related with dietary factors. Many food components have been extensively studied in relation to the development and prevention of colon cancer. However, previous studies in preclinical models have exclusively used young animals. The age of animals has not been considered in evaluation of chemopreventive effect of dietary components.

The hypothesis of this thesis was that aging may alter the cancer preventive activity of dietary components. Curcumin is a compound in the spice turmeric, powdered rhizomes of *Curcuma longa* Linn. and a well-established colon chemopreventive agent in preclinical models using young animals. This hypothesis was tested by evaluating the effect of age on the inhibition of colonic aberrant crypt foci (ACF), preneoplastic lesions of colon cancer, by dietary curcumin. Curcumin effectively reduced the number of colonic ACF in young and old rats whereas no reduction of ACF was found in middle-aged rats. This resistance of middle-aged animals to curcumin chemopreventive activity was confirmed in an ACF study with only middle-aged rats. It is important to find potential mechanisms responsible for

the resistance of curcumin chemoprevention in middle-aged animals, as it is middle to older individuals who are targeted in human clinical trials.

One consideration was that the age-related difference in curcumin absorption and metabolism might contribute to this finding. However, age did not alter the biological activity of curcumin in the liver or serum, which likely reflects absorption and metabolism of curcumin *in vivo*.

Next, arachidonic acid metabolism was explored as a potential mechanism responsible for the resistance of middle-aged rats to curcumin chemoprevention. It has been strongly believed that inhibition of cyclooxygenase-2 (COX-2) is an important colonic chemopreventive mechanism as seen in many studies investigating the relationship of non-steroidal anti-inflammatory drugs (NSAIDs) with colon cancer risk. However, in this study, curcumin, which is known to inhibit cyclooxygenase (COX) activity, increased rather than decreased transcriptional expression levels of COX-2 in the colon of both young and middle-aged rats after treatment of azoxymethane (AOM), a colon carcinogen in preclinical animal models. Moreover, in young rats, the increase of COX-2 mRNA expression in the curcumin diet group consequently increased prostaglandin E2 levels which reflect COX activity *in vivo*. This unexpected result leads to a reexamination of previous literature investigating the relationship between colorectal cancer risk and the use of NSAIDs or colonic COX-2

expression. First, it was not evident that COX-2 expression is elevated in the early stage of colon cancer. It seems that COX-2 expression is enhanced during progression of carcinogenesis. Secondly, COX-2 expression is physiologically required and has a compensatory role with COX-1 in maintaining prostaglandin levels for integrity of the gastrointestinal mucosa. No reports were found in the literature describing how AOM acutely affects expression of COX isoforms.

Interestingly, the acute time course study demonstrated that AOM did not affect the colonic mRNA levels of COX-2 but reduced colonic COX-1 mRNA expression in all age groups. These results suggest that curcumin may increase COX-2 mRNA expression in response to reduced levels of COX-1 mRNA levels in the colon. This finding may be physiologically important, as deleterious side effect of NSAIDs due to inhibition of both COX-1 and COX-2 has long been a concern with their use in chemoprevention.

Apoptosis, programmed cell death, plays a critical role in removing damaged cells upon exposure to carcinogens. Apoptosis might be a relevant mechanism to prevent initiation of colon cancer. Moreover, deregulation of apoptosis seems to contribute to the aging process. However, the means by which regulation of apoptosis contributes to aging in mitotic tissues is largely unknown although in post-mitotic tissues, it was suggested that increased susceptibility to apoptotic stimuli in

older individuals may be responsible for tissue dysfunction and aging phenotype. Therefore, a time course study of the apoptotic incidence in the colon following AOM injections in the three age groups was conducted. There were no significant age-related differences in either basal levels of apoptosis or a peak time of apoptotic incidence after carcinogen treatment. However, the peak apoptotic incidence was significantly higher in middle-aged and old rats compared to young rats. Therefore, older animals were more susceptible to AOM-induced apoptosis. One may have speculated that older animals would be more resistant to apoptosis compared to young animals, as cancer incidence increases with age, suggesting the aged are more susceptible to cancers. In fact, fewer colonic ACF were observed in older rats. The increased response of old animals to apoptotic stimuli might be beneficial in preventing cancer in the short term. However, in the long term, high incidence of apoptosis might cause loss of constituent cells and an increase of cell proliferation, increasing the likelihood of fixation of the mutation. If individuals susceptible to apoptosis are subjected to repeated exposure to carcinogens, this long-term effect of high incidence of apoptosis may be significant.

Lastly, the effect of aging on curcumin-induced apoptosis in the colons of AOM-treated and untreated rats was investigated. Curcumin increased both basal and AOM-induced apoptosis in young and old but not in middle-aged rats.

Therefore, induction of curcumin-induced apoptosis immediately after carcinogen treatment correlated with the observation of the differential chemopreventive effect of curcumin in different aged rats.

Transcriptional induction of Hsp70 by AOM was investigated. However, clear evidence that Hsp 70 had a regulatory role in induction of apoptosis by AOM and/or curcumin was not found. The family of proteins Bcl-2, IAP, and Hsp are among proteins that have a regulatory role in the induction of apoptosis. There are at least two apoptotic pathways and many different proteins that regulate apoptosis. The mitochondrial-dependent pathway appears to mediate the curcumin-induced apoptosis in young rats as caspase-9 was activated in young. However, caspase-9 was not activated by curcumin in old rats. AOM did not affect the activation of caspase-9 either in any age group. Therefore, a mitochondrial-independent pathway might be responsible for AOM-induced apoptosis and curcumin-induced apoptosis in old rats.

In conclusion, the studies in this dissertation provide evidence that aging modulates colonic chemopreventive activity of curcumin. This is the first report demonstrating the effect of aging in dietary chemoprevention. This age-related difference in chemoprevention efficacy seems to be due to a differential response to curcumin in the induction of apoptosis in different aged rats. Responsible pathways

and proteins in curcumin-induced apoptosis should be further investigated. Also, further study is needed to confirm whether this phenomenon of age-related differences in response to dietary chemoprevention occurs in humans and may contribute to the lack of agreement between preclinical studies in young animals and clinical studies with adult humans. The relationship of high susceptibility to apoptosis in older animals with high incidence of cancer should be also further investigated.

Appendices

Appendix 1. Unscheduled Sacrifices in Study 1.

Animal number	Age	Diet	Date necropsied	Reason
17	Middle-aged	CUR	11/31/01	Sacrifice delayed to make up time of delayed weight recovery following the first AOM injection.
35	Old	AIN	10/10/01	Early sacrifice for ethical reasons, deteriorating condition; Not included in incidence tables because no tissues were collected for histological evaluation except colon.
27	Old	CUR	10/30/01	Found dead; Not included in incidence tables because of moderate autolysis in all tissues.
33	Old	AIN	11/04/01	Early sacrifice for ethical reasons, deteriorating condition.
31	Old	AIN	11/31/01	Sacrifice delayed to make up time of delayed weight recovery following the first AOM injection.
25	Old	CUR	11/16/01	Early sacrifice for ethical reasons, 12 cm subcutaneous mass.
40	Old	CUR	11/29/01	Sacrifice delayed to make up time of delayed weight recovery following the first AOM injection.
39	Old	CUR	11/29/01	Sacrifice delayed to make up time of delayed weight recovery following the first AOM injection.

Appendix 2. Incidence of animals in Study 1 with macroscopic changes observed at necropsy.

Age group	Young		Middle-aged		Old	
Diet group	AIN	CUR	AIN	CUR	AIN	CUR
Tissue/ Macroscopic observation						
LUNG						
Mass, white, raised					1/8 (30)	
Consolidated, dark red					1/8 (27^{5})	
HEART						
Small					1/8 (37)	
SPLEEN						
Mass, white, raised					2/8 (30,33^{2})	
Enlarged					2/8 (27^{5}, 33^{2})	2/8 (25^{3}, 39^{4})
KIDNEY						
Discoloration, green				1/6 (17)	1/8 (31)	1/8 (40^{4})
Enlarged, bilateral					1/8 (31)	1/8 (40^{4})
Surface, granular, rough					2/8 (27^{5}, 33^{2})	
LIVER						
Nodule, raised, medial lobe			1/6 (19)	1/6 (16)		3/8 (34, 39^{4}, 25^{3})
Surface, granular, rough					3/8 (36, 27^{5}, 33^{2})	
Cyst, clear					1/8 (33^{2})	
Focus, white					1/8 (33^{2})	

Cont.: Appendix 2.

THYROID						
Enlarged					2/8 (31, 37)	
Small					1/8 (37)	1/8 (33[2])
Discolored, dark red					1/8 (31)	
ADRENAL GLAND						
Mass, dark gray					1/8 (38)	1/8 (40[4])
STOMACH						
Area, depressed, glandular mucosa					1/8 (27[5])	1/8 (29)
COLON						
Raised lesion	1/6 (10)	3/6 (1, 4, 12)	2/6 (15, 21)	3/6 (17, 22, 23)	1/8 (36)	2/8 (32, 28)
Percent affected	17%	50%	33%	50%	12.5%	25%
Discoloration, red	1/6 (11)			1/6 (18)		
TESTICLES						
Mass, gray-yellow					4/8 (30, 31, 36, 37)	5/8 (26, 29, 34, 39[4], 40[4])
Kryptorchism, bilateral						1/8 (39[4])
PITUITARY GLAND						
Enlarged			1/6 (24)			1/8 (39[4])
Discolored, red			1/6 (24)		1/8 (31)	
SKIN						
Mass, firm, white gray, subcutaneous						1/8 (25[3])

Cont.: Appendix 2.

MUSCULATURE						
Discoloration, dark red, pelvic						1/8 (28)
Discoloration, dark red, peri-ocular					1/8 (27[5])	
EYE						
Discolored, red				1/6 (16)	2/8 (27[5], 33[2])	
THORACIC CAVITY						
Hemothorax					1/8 (33[2])	
GENERAL BODY CONDITION						
Thin					1/8 (33[2])	
Total	**2**	**3**	**4**	**7**	**30**	**20**

All animals were necropsied 11/27-28/01 with the exception of animals assigned upper case numbers:

[2]Necropsied 11/4/01, [3] Necropsied 11/16/01, [4] Necropsied 11/29/01, [5] Found dead 10/30/01

Appendix 3. Incidence of animals in Study 1 with severity of common pathological age associated changes.

Age group	Young		Middle-aged		Old	
Diet group	AIN	CUR	AIN	CUR	AIN	CUR
LUNG						
Infiltrate lymphoplasmocytic	6/6	5/6	5/6	6/6	6/6	8/8
Percent affected/ Mean Severity	100% / 1.8	83% / 1.6	83% / 2.0	100% / 2.3	100% / 1.5	100% / 1.8
Microgranuloma	3/6	5/6	3/6	4/6	3/6	5/8
Percent affected/ Mean Severity	50% / 1.3	83% / 1.4	50% / 1.7	67% / 1.5	50% / 2.3	63% / 2.2
HEART						
Chronic Cardiomyopathy (CCM)	5/6	6/6	6/6	6/6	6/6	8/8
Percent affected/ Mean Severity	83% / 1.0	100% / 1.5	100% / 1.5	100% / 1.5	100% / 2.7	100% / 2.6
AORTA / LG. VESSELS / HEART VALVES						
Myxomatous degeneration	3/6	6/6	5/6	5/6	4/6	7/8
Percent affected/ Mean Severity	50% / 1.3	100% / 1.5	83% / 1.8	83% / 2.0	67% / 1.8	88% / 1.4
KIDNEY						
Chronic Progressive Nephropathy (CPN)	6/6	6/6	6/6	6/6	6/6	8/8
Percent affected/ Mean Severity	100% / 1.2	100% / 1.0	100% / 2.3	100% / 2.0	100% / 3.0	100% / 2.9

Cont.: Appendix 3.

LIVER						
Microgranuloma	0/6	1/6	0/6	2/6	2/6	3/6
Percent affected/ Mean Severity	0% / 0	17% / 1.0	0% / 0	33% / 1.0	33% / 1.0	38% / 1.7
Inflammation with necrosis	2/6	1/6	4/6	2/6	1/6	3/6
Percent affected/ Mean Severity	33% / 1.0	17% / 1.0	67% / 1.8	33% / 1.5	17% / 1.0	38% / 2.0
Infiltrate lymphoplasmocytic	1/6	0/6	1/6	2/6	5/6	3/8
Percent affected/ Mean Severity	17% / 1.0	0% / 0	17% / 2.0	33% / 1.0	83% / 2.0	38% / 1.7
Hyperplasia, biliary	0/0	0/0	6/6	6/6	5/6	8/8
Percent affected/ Mean Severity	0% / 0	0% / 0	100% / 2.5	100% / 1.7	83% / 2.6	100% / 2.5
Vacuolation, hepatocellular	3/6	3/6	6/6	4/6	5/6	7/8
Percent affected/ Mean Severity	50% / 1.0	50% / 1.7	100% / 2.8	67% / 1.5	83% / 2.2	88% / 1.3
Spongiosis hepatis (cystic degeneration)	6/6	6/6	6/6	6/6	6/6	8/8
Percent affected/ Mean Severity	100% / 1.2	100% / 1.3	100% / 2.5	100% / 2.2	100% / 3.2	100% / 2.8
Hyperplasia, nodular	0/6	0/6	0/6	1/6	0/6	2/8
Percent affected/ Mean Severity	0% / 0	0% / 0	0% / 0	17% / 3.0	0% / 0	25% / 1.5
Hyperplasia, diffuse	1/6	0/6	1/6	1/6	3/6	3/8
Percent affected/ Mean Severity	17% / 2.0	0%/ 0	17% / 2.0	17% / 2.0	50% / 2.3	38% / 2.3
Alteration, cellular, focal, eosinophilic	0/0	0/0	0/0	0/0	1/6	3/8
Percent affected/ Mean Severity	0% / 0	0% / 0	0% / 0	0% / 0	17% / 3.0	38% / 2.7
Alteration, cellular, focal, basophilic	0/0	0/0	0/0	0/0	2/6	3/8
Percent affected/ Mean Severity	0% / 0	0% / 0	0% / 0	0% / 0	33% / 2.0	38% / 2.3

Cont.: Appendix 3.

MESENTERIC LYMPH NODE						
Accumulation of macrophages	2/6	2/6	6/6	4/6	4/6	7/8
Percent affected/ Mean Severity	33% / 1.0	33% / 1.5	100% / 2.5	67% / 1.8	67% / 2.8	88% / 2.9
THYROID						
Hyperplasia, C-cell	4/6	1/6	3/6	1/6	5/6	4/8
Percent affected/ Mean Severity	67% / 1.5	17% / 1.0	50% / 1.7	17% / 1.0	83% / 1.6	50% / 2.5
ADRENAL GLAND						
Vacuolation, Zona fasciculata	0/6	0/6	0/6	2/6	2/6	3/8
Percent affected/ Mean Severity	0% / 0	0% / 0	0% / 0	33% / 1.5	33% / 1.0	38% / 2.0
STOMACH						
Infiltrate lymphoplasmocytic	0/6	4/6	0/6	2/6	1/6	4/8
Percent affected/ Mean Severity	0% / 0	67% / 1.3	0% / 0	33% / 1.0	17% / 1.0	50% / 1.3
Dilation, glandular	0/6	1/6	2/6	3/6	6/6	5/8
Percent affected/ Mean Severity	0% / 0	17% / 1.0	33% / 1.5	50% / 1.0	100% / 1.7	63% / 2.2
DUODENUM						
Infiltrate lymphoplasmocytic	0/6	1/6	1/6	1/6	1/6	3/8
Percent affected/ Mean Severity	0% / 0	17% / 1.0	17% / 2.0	17% / 1.0	17% / 1.0	38% / 1.3
Vacuolation, villi, lamina propria	3/6	1/6	2/6	0/6	1/6	0/8
Percent affected/ Mean Severity	50% / 2.7	17% / 2.0	33% / 1.5	0% / 0	17% / 3.0	0% / 0

Cont.: Appendix 3.

PANCREAS						
Atrophy, acinar cells	1/6	3/6	2/6	1/6	2/6	3/8
Percent affected/ Mean Severity	17% / 1.0	50% / 1.7	33% / 2.0	17% / 1.0	33% / 1.5	38% / 1.3
JEJUNUM						
Vacuolation, villi, lamina propria	1/6	1/6	2/6	1/6	0/6	1/8
Percent affected/ Mean Severity	17% / 1.0	17% / 1.0	33% / 2.5	17% / 2.0	0% / 0	12.5% / 2.0
ILEUM						
Vacuolation, villi, lamina propria	3/6	1/6	1/6	0/6	0/6	0/8
Percent affected/ Mean Severity	50% / 2.3	17% / 1.0	17 % / 0	0% / 0	0% / 0	0% / 0
SMALL INTESTINES						
Vacuolation, epithelial, villi and lamina propria	5/6	2/6	4/6	1/6	1/6	1/8
Percent affected/ Mean Severity	83%/ 2	33%/ 1.9	68%/ 2	17%/ 2	17%/ 3	12.5%/ 2
COLON	6/6	6/6	6/6	6/6	6/6	7/8
Crypt drop out						
Percent affected/ Mean Severity	100% / 2.7	100% / 2.0	100% / 2.2	100% / 2.2	100% / 2.0	88% / 2.3
Inflammation, interstitial, focal	6/6	6/6	6/6	5/6	6/6	8/8
Percent affected/ Mean Severity	100% / 1.3	100% / 1.2	100% / 1.5	83% / 1.6	100% / 1.2	100% / 1.5
Infiltrat, lymphoplasmocytic, lamina propria	2/6	5/6	5/6	6/6	5/6	8/8
Percent affected/ Mean Severity	33% / 1.5	83% / 1.4	83% / 1.4	100% / 1.5	83% / 1.8	100% / 1.6

Cont.: Appendix 3.

TESTES						
Hyperplasia, interstitial cell	2/6	0/6	5/6	5/6	6/6	6/8
Percent affected/ Mean Severity	33% / 1.0	0% / 0	83% / 1.6	83% / 1.8	100% / 1.8	75% / 2.7
EPIDIDYMIS						
Vacuolation, epithelial, basophilic, cytoplasmic	0/6	1/6	4/6	1/6	3/5	5/8
Percent affected/ Mean Severity	0% / 0	17% / 2.0	67% / 1.8	17% / 2.0	60% / 2.7	63% / 2.0
PROSTRATE GLAND						
Inflammation	5/6	4/6	6/6	3/6	4/5	7/7
Percent affected/ Mean Severity	83% / 2.2	67% / 2.3	100% / 1.8	50% / 3.0	80% / 2.8	100% / 2.7
Hyperplasia, epithelial	5/6	5/6	6/6	3/6	4/5	6/7
Percent affected/ Mean Severity	83% / 1.6	83% / 2.0	100% / 2.0	50% / 2.0	80% / 1.5	86% / 2.2
SEMINAL VESICLE						
Hyperplasia, epithelial	5/6	2/6	2/6	2/6	3/5	4/7
Percent affected/ Mean Severity	83% / 2.0	33% / 1.5	33% / 2.0	33% / 2.0	60% / 2.3	57% / 2.5
COAGULATING GLAND						
Hyperplasia, epithelial	4/6	4/6	4/6	3/6	3/5	4/7
Percent affected/ Mean Severity	67% / 1.5	67% / 2.0	67% / 1.8	50% / 1.3	60% / 2.3	57% / 2.3

Cont.: Appendix 3.

POLYARTERITIS NODOSA Affecting multiple organs						
Stomach						1/8
Percent affected/ Mean Severity						13% / 2.0
Pancreas			1/6			
Percent affected/Mean Severity			17% / 3.0			
Testis					1/6	
Percent affected/Mean Severity					17% / 2.0	

Appendix 4. Incidence of animals in Study 1 with severity of small intestinal lamina propria vacuolation.

Age group	Young		Middle-aged		Old	
Diet group	AIN	CUR	AIN	CUR	AIN	CUR
Small intestines						
Vacuolation, villi, lamina propria	5/6	2/6	4/6	1/6	1/6	1/8
Percent affected/ Mean Severity	83% / 2	33% / 1.9	68% / 2	17% / 2	17% / 3	12.5% / 2

Appendix 5. Incidence of animals in Study 1 with severity of hepatocellular hyperplasia (nodular and diffuse) and altered hepatocellular foci.

Age group	Young		Middle-aged		Old	
Diet group	AIN	CUR	AIN	CUR	AIN	CUR
Liver						
Hyperplasia, hepatocellular	1/6	0/6	1/6	1/6	3/6	5/8
Percent affected/ Mean Severity	17% / 2	0% / 0	17% / 2	17% / 3	50% / 2.3	63% / 2
Alteration, hepatocellular, focal	0/6	0/6	0/6	0/6	2/6	5/8
Percent affected/ Mean Severity	0% / 0	0% / 0	0% / 0	0% / 0	33% / 2.25	63% / 2.2

Appendix 6. Incidence of animals in Study 1 with neoplastic lesions.

Age group	Young		Middle-aged		Old	
Diet group	AIN	CUR	AIN	CUR	AIN	CUR
Lung						
Carcinoma	0/6	0/6	0/6	0/6	1/6	0/8
Percent affected	0%	0%	0%	0%	17%	0%
Hemo- lymphocytic system						
Lymphoma	0/6	0/6	0/6	0/6	1/6	0/8
Percent affected	0%	0%	0%	0%	17%	0%
Spleen						
Histiocytic Sarcoma	0/6	0/6	0/6	0/6	1/6	0/8
Percent affected	0%	0%	0%	0%	17%	0%
Thyroid Glands						
Adenoma	0/6	0/6	0/6	0/6	0/6	4/8
Percent affected	0%	0%	0%	0%	0%	50%
C-cell Carcinoma	0/6	0/6	0/6	0/6	2/6	0/8
Percent affected	0%	0%	0%	0%	33%	0%
Adrenal Glands						
Pheocromocytoma	0/6	0/6	0/6	0/6	1/6	0/8
Percent affected	0%	0%	0%	0%	17%	0%
Adenoma, Zona reticularis	0/6	0/6	0/6	0/6	1/6	0/8
Percent affected	0%	0%	0%	0%	17%	0%
Pancreas						
Adenoma, islet cell	0/6	0/6	0/6	0/6	1/6	0/8
Percent affected	0%	0%	0%	0%	17%	0%
Testes						
Adenoma, interstitial cell	0/6	0/6	0/6	0/6	5/6	6/8
Percent affected	0%	0%	0%	0%	83%	75%
Pituitary Gland						
Adenoma, pars distalis	0/6	0/6	1/6	0/6	2/6	1/8
Percent affected	0%	0%	17%	0%	33%	12.5%
Skin						
Fibroadenoma	0/6	0/6	0/6	0/6	1/6	0/8
Percent affected	0%	0%	0%	0%	17%	0%
Total	0	0	1	0	16	11

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