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By

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ABSTRACT

SOY REDUCES COLON CANCER RISK IN HUMANS AND RATS

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Three studies were conducted to determine 1) the influence of age and history of colonic polyps, cancer or FAP on colonic epithelial cell proliferation indices in humans, 2) the efficacy of soy protein isolate (SPI) supplements to reduce intermediate biomarkers of colon cancer (CC) risk in humans at risk for CC, and 3) the influence of soy products on development of aberrant crypt foci (ACF) and on colonic epithelial cell proliferation in AOM-initiated rats. In the first study, subjects (N=46) were grouped based on age and clinical history of polyps, FAP and CC. Colon biopsies were obtained by colonoscopy and cell proliferation was measured by proliferating cell nuclear antigen (PCNA) and Ki-67 immunohistochemistry (IHC) and relative PCNA protein levels by slot-blot quantification. PCNA labeling index (LI), PCNA proliferating zone (PZ), Ki-67 PZ and PCNA protein levels increased with age and history of polyps, FAP or CC ($P < 0.05$). The PCNA LI and PZ, Ki-67 PZ, and PCNA protein in colonic mucosa were highly correlated with each other and were highly correlated with risk of CC which indicates that these biomarkers have utility as intermediate biomarkers in chemoprevention studies.

The second experiment was a double-blind, prospective study designed to determine if consumption of one of two supplements containing 38 g/d of SPI with 58 mg of total genistein or 40 g/d of calcium caseinate for one year would influence colonic epithelial cell proliferation as measured by PCNA and Ki-67 labeling and PCNA protein expression in colonic epithelial cells. Colonic biopsies from were obtained from subjects

(N=42; age = 34-70; previous history of adenomatous polyps or CC) before and after supplementation and used for IHC detection of PCNA and Ki-67 antigens in colon crypts and for determination of relative PCNA protein expression by slot-blotting. There were statistically significant reductions in both PCNA LI and PCNA PZ in colonic mucosa of subjects that consumed the soy supplements, but not in subjects who consumed casein at the end of the study period. The relative expression of PCNA protein in colonic mucosa was significantly reduced by consumption of either of the soy supplements, but was not influenced by consumption of the casein supplement.

The third study investigated the potential of different soy products with varying levels of phytochemicals to reduce aberrant crypt foci (ACF) development and colonic epithelial cell proliferation in carcinogen-initiated rats. The dietary treatments (n = 15 rats/treatment) were full fat soy flakes (SFK), de-fatted soy flour (SFL), soy concentrate (SC), SC plus 150 mg/kg genistein (GEN), and SC plus 5 g/kg calcium (CAL). After 12 weeks of dietary treatment, rats consuming SC had the greatest ($P < 0.05$) numbers of total ACF, large ACF and total aberrant crypts among all treatment groups. The GEN and CAL treatments resulted in the lowest ($P < 0.05$) numbers of ACF. Rats fed SFK had the lowest ($P < 0.05$) PCNA LI and PZ. Rats fed SFL and SFK had significantly lower levels of PCNA protein when compared to rats consuming SC, GEN, or CAL diets. Quantified PCNA protein expression positively correlated with LI and PZ ($r = 0.81$, $r = 0.65$, respectively at $P<0.0001$) analyses of PCNA by IHC. In conclusion, the reductions we observed in colonic epithelial cell proliferation in humans and animals consuming soy in these studies are characteristic of alterations that would be associated with an overall decrease in risk for CC.

Dedicated to

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LIST OF ABBREVIATIONS

ACF.....Aberrant Crypt Foci

ADME.....Absorption, Distribution, Metabolism and Excretion

AEC.....Aminoethyl Carbazole

AICR/WCRF.....American Institute for Cancer Research/World Cancer Research Fund

AIN93G.....American Institute of Nutrition 93 Growth diet

AOM.....Azoxymethane

AP.....Adenomatous Polyps

APC.....Adenomatous Polyposis Coli

AUC.....Area Under the Curve

BBI.....Bowman-Birk Inhibitor

BMI.....Body Mass Index

BSA.....Bovine Serum Albumim

CAL.....Calcium

CC.....Colon Cancer

CH.....Crypt Height

COX.....Cyclooxygenase

DCC.....Deleted in Colon Cancer

DM.....Diabetes Mellitus

DMH.....Dimethylhydrazine

EDTA.....Ethylene Diamine Tetra Acetic acid

EGF.....Epidermal Growth Factor

FAP.....Familial Adenomatous Polyposis

GEN.....Genistein

hMLH1.....Human Mut-L Homologue 1

HNPCC.....Hereditary Non-Polyposis Colorectal Cancer

HRT.....Hormone Replacement Therapy

IHC.....Immunohistochemical

LEF.....Lymphoid Enhance Factor

LI.....Labeling Index

MAPK.....Mitogen Activated Protein Kinase

MOPS.....4-Morpholine Propane Sulfonic Acid

NSAID.....Non Steroidal Anti-Inflammatory Drugs

PBBI.....Purified Bowman-Birk Inhibitor

PBS.....Phosphate-Buffered Saline

PCNA.....Proliferating Cell Nuclear Antigen

PKC.....Protein Kinase C

PMSF.....Phenylmethylsulphonylfluoride

PVDF.....Polyvinylidenefluoride

PZ.....Proliferation Zone

RR.....Relative Risks

SAS.....Statistical Analysis System

SC.....Soy Concentrate

SDS.....Sodium Dodecyl Sulphate

SEB.....Surrogate End- point Biomarkers

SEM.....Standard Error of the Mean

SFK.....Soy Flakes

SFL.....Soy Flour

SPI.....Soy Protein Isolate

TBS.....Tris-Buffered Saline

TCF.....T Cell Factor

TGF.....Transforming Growth Factor

CHAPTER I. INTRODUCTION

In the United States, colon cancer (CC) is the third most common form of cancer in terms of incidence and second most common cause of cancer mortality (American Cancer Society, 2005). It is estimated that 66-75% of CC incidence could be prevented by adequate diets and with appropriate changes made to current dietary habits and lifestyle practices (AICR/WCRF, 1997). Increasing the consumption of foods that contain anti-carcinogenic phytochemicals is emphasized as a means to reduce incidence of many diet-related cancers such as CC. Even though there is no strong correlation between any particular food and CC risk, many cross-cultural epidemiological studies indicate that consumption of soy foods may contribute to the relatively low rates of colon, breast and prostate cancers observed in east Asian countries such as China and Japan (Messina et al., 1994; Barnes, 1998; Messina, 1999; Birt et al., 2001).

The development of CC is a process involving multiple steps, and often is a consequence of an intimate, but only moderately understood, interplay between genetic and environmental factors (Fearon and Vogelstein, 1990). As the colorectal epithelium progresses from a normal phenotype to one that is hyperproliferative, multiple genetic and epigenetic alterations occur, including activation of proto-oncogenes, inactivation of tumor suppressor genes, and mutations in mismatch repair genes leading to malignancy (Fearon and Jones, 1992).

Dietary factors can potentially affect each step of tumorigenesis, thereby modulating the above-mentioned genetic and epigenetic changes. The process of using natural or synthetic chemical compounds to reverse, suppress, and/or prevent progression to invasive cancer is referred as "chemo-prevention" (Lippman et al., 1994). Cancer

chemo-prevention studies using animal models provide for strict control of variables, but inherent species differences can be a problem, precluding direct extrapolations of observations from animal studies to humans. Previous studies in humans have tested the ability of candidate dietary components for their utility as chemo-preventive agents in colonic carcinogenesis with little success (Einspahr et al., 1997; Barnes et al., 1998; Krishnan et al., 1998). Cancer intervention trials using human subjects are exceedingly difficult to conduct because of the slow progressive nature of carcinogenesis and the large numbers of subjects required to achieve adequate statistical power (Kim and Mason, 1996; Pfeiffer et al., 2003).

In the last decade, several strategies have been employed to circumvent the problems associated with conducting cancer intervention trials in humans. One strategy is to study those individuals who are at high risk (age, history of polyps, FAP) for developing CC to determine whether a certain chemo-preventive agent can suppress or prevent cancer development. Another strategy is to use early or intermediate biomarkers as surrogate end points for cancer risk rather than relying on occurrence or recurrence of CC (Sharma et al., 2001; Garcea et al., 2003). Alterations in mucosal proliferation indices, aberrant histopathologic features, abnormal immunologic and biochemical markers, and more recently, alterations of molecular markers have been extensively studied for their applicability and utility as intermediate markers in colorectal cancer chemoprevention trials (Deschner, 1988; Lipkin, 1988; Singh et al., 1993; Bostick et al., 1995; Greenwald et al., 1995; Toribara and Sleisenger, 1995; Mark, 1996; Thigalingam et al., 1996; Takayama et al., 1998; Gryfe et al., 1999).

Recognizing carcinogenesis as a multi-step process, one of the first indications of preneoplastic changes in colorectal mucosa is increased epithelial cell proliferation. Actively dividing cells produce a number of unique proteins. Proliferating cell nuclear antigen (PCNA) is one protein whose level of synthesis correlates directly with the rate of cellular proliferation and DNA synthesis. Previous research indicates that PCNA is a reliable biological marker for proliferative activity in colonic crypts (Malecka-Panas et al., 1997; Shpitz et al., 1997; Barnes et al., 1998). PCNA expression correlates well with that of other proliferation markers like bromo-deoxyuridine (BrdU) and Ki-67 antigen expression in both normal and neoplastic colonic epithelium (Kubota and Kino, 1995; Yerly-Motta et al., 1999; Wu et al., 2000; Díez et al., 2003). Unfortunately, the immunohistochemical (IHC) techniques used to determine cell proliferation using PCNA or other endogenous markers are relatively long and laborious. Hence, there is a need for a rapid, reliable and valid method of measuring PCNA protein levels in colonic mucosa that could be utilized in human studies to test for potential chemo-preventive agents. A simpler and more rapid method for PCNA protein quantification may also have utility in the clinical assessment of colon biopsy specimens.

To date, there has been no published research which compares PCNA and Ki-67 proliferation indices in persons of widely varying ages who have no history of colonic diseases. A study was designed to assess and directly compare PCNA and Ki-67 proliferation indices (using IHC) in colonic mucosa biopsies obtained from persons who differed in age (without any history of colonic diseases) and in persons with known risk factors (history of adenomas, familial adenomatous polyposis and history of CC) for colon cancer development. The second objective of this study was to use an

immunoblotting method to determine relative levels of PCNA protein in colonic epithelial cells and determine if PCNA protein expression correlated with proliferation indices based on IHC techniques.

Epidemiological studies have reported that more frequent consumption of vegetable protein, including soy protein, is associated with reduced risk for CC. There are no published studies on the effects of soy protein consumption on colonic epithelial cell proliferation in humans. A second study was then conducted to determine the potential chemo-preventive activity of soy protein isolate in humans at risk for CC. This clinical trial was designed to determine if consuming 38 g of soy protein isolate supplement per day for one year would reduce CC risk as reflected by changes in colonic epithelial cell proliferation patterns and quantified PCNA levels (by immunoblotting method) in colonic mucosa. A secondary objective was to determine if adding or removing calcium from the soy protein isolate supplement would alter the impact of soy on colonic epithelial cell proliferation in humans.

Soybeans and soybean-based foods are a good source of several phytochemicals, including isoflavones, phytosterols, saponins, protease inhibitors and phytate that have been reported to have potential anticancer properties (Messina and Bennink, 1998). Therefore, the third study was conducted to determine if soy-containing diets having differing concentrations of phytochemicals (especially isoflavones) can reduce preneoplastic lesions and indices of colonic epithelial cell proliferation (as assessed by PCNA and Ki-67 IHC in colonic tissue and by PCNA protein quantification (by immunoblotting) in rats initiated with a colon carcinogen (azoxymethane). A further objective was to determine if changes in colonic epithelial cell proliferation patterns (as

influenced by dietary treatments) correlated with the numbers of preneoplastic lesions detected in the colons of rats treated with azoxymethane and subjected to dietary treatments differing in soy preparations and phytochemical contents. The final objective of this study was to determine if dietary calcium concentration (1 or 5 g/kg of diet) would influence preneoplastic lesion development and markers of colonic epithelial cell proliferation in these rats.

A reduction in colonic epithelial cell proliferation, a reduced size of the zone of proliferation in colonic crypts, and a decrease in quantified PCNA levels in the colon mucosa (of both animals and humans) after consumption of soy compounds would suggest that one or more phytochemicals present in soy is responsible for altering the cell proliferation pattern. Thus, these studies will demonstrate if chemo-preventive agents in soy inhibit colonic epithelial cell proliferation as reflected by changes in PCNA and Ki-67 expression and total quantity of PCNA protein in colonic mucosa, alterations that would be associated with an overall decrease in risk for CC.

CHAPTER II. REVIEW OF LITERATURE

A. Epidemiology: Incidence patterns and distribution of colon cancer

Cancer of the colon and rectum is the third most common incident cancer and cause of death from cancer, throughout the world (AICR/WCRF, 1997). In 2000 alone, an estimated 945,000 were diagnosed worldwide, accounting for 9.4% of all new cases of cancer (Parkin, 2004). In the U.S., colorectal cancer is the third most common incident cancer and second most common cause of mortality (American Cancer Society, 2005). The incidence of colorectal cancer in the U.S. has been declining since 1998 (American Cancer Society, 2005). However, worldwide incidence of and deaths from colon cancer (CC) are generally increasing, mostly in the developed countries and in urban areas of the developing countries (Parkin, 2004). High- risk areas include North America, Europe and Australia, New Zealand (Parkin, 2004) accounting for over 63% of the total global incidence. Incidence is also now increasing in what was considered as low risk areas such as Central and South America, Asia and Africa (AICR/WCRF, 1997).

Worldwide incidence of colon cancer is similar in both men and women (Parkin, 2004). In the United States, colorectal cancer incidence and mortality rates are more than 35% higher in men than in women (American Cancer Society, 2005). In 2002, the age-adjusted incidence rates for CC were 62.1 and 46.0 cases per 100,000 population in men and women, respectively. The age-adjusted rates for rectal cancer were 24.8 and 17.4 cases per 100,000 population in men and women, respectively (Ries et al., 2005). According to migrant and temporal trend studies colorectal cancers are largely determined by environmental exposures. Colorectal cancer rates have increased in the migrants that moved from low risk to high- risk countries reflecting the host countries

rates within one or two generations (Parkin, 2004). There is at least a 25-fold variation in colorectal cancer worldwide (Parkin, 2004). Regional difference in incidence rates may be explained, in large part, by dietary and other environmental differences. Colorectal cancer has also long been known to occur more frequently in certain families (Macklin, 1960) and there are several rare genetic syndromes that carry a markedly elevated risk (Gardner, 1951; Veale, 1965; Utsunomiya and Lynch, 1990). Colorectal cancer is thus casually related to both genes and environment.

B. Genetic basis for colorectal carcinogenesis

1. The multistage hypothesis

Colon cancer development is a multi-stage process in which transformed cells progress to precancerous lesions (e.g. adenomatous polyps) and, ultimately, carcinomas during a period spanning several years to decades. The gastrointestinal epithelium is a complex microenvironment, made up of at least five interrelated cell types and characterized by a tightly regulated program of cell proliferation, maturation, differentiation and apoptosis. Carcinogenesis in colonic mucosa is likely the result of successive accumulation of multiple genetic mutations, resulting in transformed phenotype and eventual progression to invasive cancer. Vogelstein et al. (1988) described specific mutations in colorectal cancer and defined their relationship to the adenoma-carcinoma sequence. This led to wide acceptance of the multistep hypothesis as basis for malignant transformation and gave a genetic perspective to the processes of colon tumor initiation, promotion and progression.

Fearon and Vogelstein (1990) in their genetic model, indicate a hyperproliferative colonic epithelium as the first step toward CC development. People at high risk for colon cancer (old age, presence of adenomatous polyps or familial adenomatous polyposi [FAP]) have increased mucosal cell proliferation throughout their colon. Gene mutations or deletions accompany the promotion from a hyperproliferative normal crypt to a small adenoma. In this initiation stage, the adenomatous polyposis coli gene (APC) acts as a gatekeeper of colonic epithelial proliferation. One key function of the normal APC protein in colonic epithelial cells is to form a multi-protein complex (with axin/conductin and glycogen synthase kinase-3β) that binds to cytosolic β-catenin and targets it for destruction via the ubiquitin-proteasome pathway (Easwaran et al., 1999). APC gene mutations typically result in the production of truncated APC protein that is unable to bind to β-catenin, thereby allowing the cytosolic accumulation of β-catenin. When β-catenin levels are elevated, it can translocate to the nucleus, bind to T cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors, and stimulate transcription of a wide variety of gene products via activation of the Wnt signaling pathway (Ilyas and Tomlinson, 1997). Thus, APC gene mutation is permissive for persistently elevated concentrations of β-catenin, which ultimately results in abnormal cell proliferation, differentiation and migration in the colonic mucosa. Mutations of the APC gene are a frequent and early event of sporadic colon tumorigenesis (Luchtenborg et al., 2004).

Another common genetic event in colon carcinogenesis is mutation of the K-ras gene (Fearon and Vogelstein, 1990). Transformation to the cancerous state (adenocarcinoma) is frequently associated with the loss of the DCC (Deleted in Colon Cancer) gene and/or loss of the p53 gene. The p53 gene encodes for a protein having a

significant homology to cell adhesion family of proteins. This protein can also block DNA-damaged cells from progressing to the S phase of the cell cycle thereby acting as a check point to allow for adequate DNA repair before permitting another round of cell division. Mutated p53 protein binds to and inactivates wild type p53 protein. Mutation and or deletion of p53 gene plus a K-ras mutation is sufficient to transform a normal cell to a cancerous one. Approximately 60 - 85% of colon tumors have mutated p53 genes (Baker et al., 1989; Yamaguchi et al., 1994a; Yamaguchi et al., 1994b).

The DCC gene encodes a plasma membrane protein that is believed to function in cell-cell communication and/or cell-substrate interactions (Cho et al., 1994). As mucosal cells travel toward the flat mucosa of the crypt, the DCC encoded protein may pass signals from the submucosa directing cells to undergo terminal differentiation. There is a strong relationship between lack of DCC expression, lack of cell differentiation, and tumorigenesis (Hedrick et al., 1994). Approximately 75% of colorectal tumors and 40% of adenomas with foci of carcinomatous transformation have a mutated or missing DCC gene (Fearon et al., 1990).

2. Familial adenomatous polyposis (FAP)

Familial adenomatous polyposis is an autosomal dominantly inherited disease, which was first described by Bussey in 1975. This condition is responsible for less than 1% of all colorectal cancers and has a prevalence of about 1 in 10,000 (Bishop and Thomas, 1990; Bisgaard et al., 1994). The gene responsible for FAP was shown to be present on chromosome 5 by Leppert et al., in 1987. This gene was later identified and cloned and now referred to as the APC or Adenomatous Polyposis Coli gene (Groden et

al., 1991; Nishisho et al., 1991). A germ-line mutation in the APC gene causes FAP, which is characterized by a permanent imbalance in cell proliferation leading to hyperproliferative epithelium preceding the formation of adenomas. Individuals with FAP inherit a single mutated allele of the APC gene that acts in a dominant negative manner leading to development of dozens to hundreds of colonic adenomas at a very young age.

3. Hereditary non-polyposis colorectal cancer (HNPCC)

HNPCC is a heritable dominant condition with a high degree of gene penetrance. A family of four DNA mismatch repair genes have been identified to be responsible for the development of HNPCC (Papadopoulos et al., 1994). They are regarded as oncosuppressor genes, a second copy of one of the four genes being inactivated either by mutation or loss of the gene. The subsequent breakdown of the DNA repair mechanisms leads to an accelerated pathway of neoplastic evolution in which gene activation is preferentially mediated by somatic mutation as opposed to an allelic loss (Leach et al., 1993).

C. Morphology of colorectal neoplasia precursor and associated lesions of colorectal cancer

Colon carcinogenesis is a multi-step process in which initiated cells progress through various stages (e.g. ACF, microadenomas, adenomatous polyps), ultimately leading to carcinoma in situ and invasive colon cancer. The various precursor and

associated lesions which differ morphologically from the normal colorectal mucosa are discussed in the following paragraphs.

1. Hyperproliferation and Aberrant Crypt Foci

Increased colorectal crypt cell proliferation, a well as an expansion of the proliferative zone toward the luminal surface of the crypts have been regarded as an early indicators of future risk for the development of colon tumors (Friedman, 1985; Lipkin, 1988; Fearon and Vogelstein, 1990; Srivastava et al., 2001). Hyper proliferation has been observed in adenomas as compared with benign epithelium, with the highest proliferation seen in adenomas with high grade dysplasia. In addition, a shift of proliferation toward the luminal surface of the epithelium occurs in adenomas as well as aberrant crypt foci (ACF). ACF were first described by Bird, in chemically induced rodent models of colon carcinogenesis when the whole mounts of colon tissue was stained with methylene blue and examined under low power magnification (Bird, 1987; McLellan and Bird, 1988). These lesions have been also identified in macroscopically normal human colonic mucosa (Pretlow et al., 1991; Roncucci et al., 1991). ACF are described as usually having larger than normal crypts, with large cells microscopically elevated above the mucosal surface, and often show increased branching and proliferation. They also have been reported to contain cells displaying features ranging from almost normal to dysplastia, with a variety of changes in mucin production and goblet cells, suggesting heterogeneity at an early stage of carcinogenesis (McLellan et al., 1991a; McLellan et al., 1991b; Caderni et al., 1995; Pretlow, 1995). Hyperproliferative lesions described in the earlier studies could have been instances of ACF, or at least included ACF. Mutations of K-ras,

APC, and some p53 genes occur in human ACF and in chemically induced ACF in experimental animals (Smith et al., 1994; Yamashita et al., 1995).

The numbers of ACF can be influenced by diet and other factors in the colonic environment. Studies have shown stimulation of ACF by exposure to secondary bile acids and suppression by primary bile acids. In chemically-induced rodent models of CC dietary fiber, beta-carotene and other retinoids, soy phytochemicals (genistein, inositol hexaphosphate, protease inhibitors, saponins), calcium, aspirin and other NSAIDS, all have been demonstrated to inhibit ACF formation (Rao et al., 1992; Thorup et al., 1994; Pretlow, 1995; Wargovich et al., 1995a; Wargovich et al., 1995b). Although there is no direct evidence for ACF transitioning to micro-adenomas, adenomas or carcinomas, they are being regarded as early morphological changes in colorectal neoplasia. Jen et al. (1994) reported that only those ACF having morphology similar to micro-adenomas and APC gene mutations were likely to progress to carcinomas. ACF also appears to develop in response to certain environmental agents such as dietary factors, alcohol and smoking, which in some individuals produce non-neoplastic hyperproliferative lesions such as hyperplastic polyps and may, depending on nature of mutations that occur, result in development of colorectal tumors. A better understanding of the morphologic and molecular heterogeneity of ACF is needed to help elucidate the early phases of colorectal neoplasia.

2. Colo-rectal Micro-Adenomas

These were first recognized in 1991 (Roncucci et al.) during a microscopical examination of ACF. As mentioned earlier, there is no known relationship between ACF

and micro-adenomas but it is possible that a minority of ACF are microadenomas at the time of onset, and that some of these may grow to become endoscopically recognizable adenomas. The nature of the genetic change in ACF may be an important determinant of their ultimate progression to other lesion types. Non-neoplastic hyperplastic foci may develop into hyperplastic polyps, whilst those foci which also have APC mutations are likely to develop into micro-adenomas, and some of which then progress into macro-adenomas (Jen et al., 1994). Micro-adenomas have now become an important link in the morphology of colorectal neoplastic events as well as in the concept of "de-novo" colorectal cancer.

3. Hyperplastic Polyps

Hyperplastic polyps endoscopically look similar to adenomatous polyps, however have a distinct histology. Hyperplastic polyps range in size from 1 – 40 mm and are more frequent in the distal than the proximal colon (Ferrandez et al., 2004). Until recently, hyperplastic polyps were not considered likely to progress to colorectal cancer. There was indirect evidence of a relationship of hyperplastic polyps to adenomas and colorectal cancers due to similarities in their distribution (Isbister, 1993). Furthermore, hyperplastic polyps in the distal large bowel had been found to be strong indicators for the presence of colorectal adenomas in the proximal large bowel (Nusko et al., 1994; Van Stalk et al., 1994). Recent studies indicate that hyperplastic polyps are morphologically similar to serrated adenomas and serrated adenocarcinomas and are likely to be biologically related. Chan et al. (2003) found that acquisition of a BRAF mutation was associated with the progression of hyperplastic polyps to serrated adenomas, whereas acquisition of K-ras

mutations was associated with the progression of hyperplastic polyps to an admixed hyperplastic polyp/adenoma morphology. Hawkins and Ward (2001) demonstrated that expression of human mut-L homologue 1 (hMLH1) protein, a DNA mismatch repair enzyme, was lost in benign hyperplastic polyps and serrated adenomas obtained from 10 of 13 subjects previously diagnosed with microsatellite-unstable CC. This loss of hMLH1 protein expression was associated with methylation of the promoter region of the hMLH1 gene, and concluded that methylation of the hMLH1 promoter within these neoplastic cell populations may be a critical step in the progression to carcinoma (Hawkins and Ward, 2001). Hyman et al. (2004) conducted a prospective study to assess the risk of patients having hyperplastic polyps to develop CC, and concluded that these subjects are, in fact, at high risk for developing CC.

4. Adenomas

These are benign tumors of the intestinal epithelium and in Western populations are considered as the major precursor of colorectal cancer. The monoclonal origin of adenomas, that is, their commencement from one stem cell was first established by Fearon et al. (1987). Current evidence suggests that adenomas commence in a subset of proliferative lesions such as ACF, becoming first a micro-adenoma and then a visible adenoma. Adenomas are classified according to their appearance as protuberant or polypoid, or as flat adenomas. Microscopically, adenomas are classified as tubular, villous or tubulovillous. Polypoid adenomas are the most common type of adenoma in Western populations and their evolution appears to be associated with both p53 and K-ras mutations (Fujimori et al., 1994; Yukawa et al., 1994).

Macroscopically flat adenomas were first recognized by Muto and co-workers in Japan (Muto et al., 1985; Minamoto et al., 1994). In Western populations flat adenomas are much less frequent than polypoid adenomas, although their frequency is uncertain as they can be difficult to identify during colonoscopy (Matsumoto et al., 1994). They are probably more common in the distal than in proximal colon, suggesting environmental exposures to be important in their etiology. Flat adenomas also tend to occur at an earlier age than polypoid adenomas and are more aggressive, both histologically and clinically, regarding malignant potential. Furthermore, ras mutations are not expressed in flat adenomas (Fujimori et al., 1994; Yukawa et al., 1994) but APC gene mutations have been detected (van Wyk et al., 1999). An epidemiologic overview of these biologic differences suggest that quantitative rather than qualitative differences of inherited and environmental exposures are responsible for the different frequency of flat compared to polypoid adenomas in Japan versus Western populations.

5. Severe Dysplasia, Carcinoma In Situ

Histologic changes intermediate between normal colorectal mucosa and a neoplastic lesion are termed as "dysplasia", and these changes have been graded into mild or low-grade and severe or high-grade dysplasia (O'Brien et al., 1990). The terms "severe dysplasia" or "high-grade dysplasia" are often preferred to terms "carcinoma in situ" or "intramucosal carcinoma" because these lesions probably lack the ability to invade and metastasize, as it requires further genetic and immunologic changes.

6. "De Novo" Carcinoma

These are small non-polypoid cancerous lesions that account for one-third of the incident cases in Western populations (Kuramoto and Oohara, 1995). There has been some evidence to believe these cancers originate from normal colorectal mucosal cells ("de-novo"), and other evidence suggests that these cancers evolve from flat adenomas. The flat adenoma-adenocarcinoma sequence has also been documented in chemically induced colon tumors in rats, and about one-third of all neoplasms were of the flat variety, a proportion similar to "de-novo" cancers in Western populations (Rubio and Shetye, 1994).

7. Invasive Colorectal Cancer

When cancer cells penetrate the muscle layer, they are regarded as invasive cancers which are able to spread, metastasize and cause premature death. The ability to become invasive and then metastasize appears to involve further somatic mutations and possibly other host-defense mechanisms which at present are poorly understood.

D. Role of environmental factors in colorectal cancer

Diet has long been regarded as the most important environmental influence on colorectal cancer. Multiple confounding interactions among dietary constituents have made it difficult to determine the relationship between diet and colorectal cancer. Despite this, there is a growing body of evidence from both human epidemiological studies and animal studies that have linked consumption of several dietary components to colorectal cancer risk. Ever since Burkitt (1969) noted an inverse association between fiber intake

and CC incidence, several prospective and case-control studies have confirmed the relationship between fiber consumption and colorectal neoplasia (Giovannucci et al., 1992). Increased risk for CC has been associated with high dietary fat intakes, particularly that of saturated fats present in red meat (Newmark et al., 1991; Potter, 1992; Giovannucci et al., 1994). Numerous epidemiologic studies have reported that populations having high vegetable and fruits intakes have a decreased incidence of CC (Potter, 1993). A number of additional dietary agents, such as vitamins, micronutrients, and other minor dietary components have been suggested to lower risk for CC. Because the consumption of fiber, fat, red meat, fruits, vegetables, antioxidants, or micronutrients are all closely linked, it is difficult to point to any one of these as a causative or protective agent for CC.

Equally difficult is to distinguish between energy intake, and intake of fat (the most energy dense constituent of the diet) when studying their potential relationships with CC risk. Cohort studies (Willett et al., 1990; Bostick et al., 1994; Giovannucci et al., 1994; Goldbohm et al., 1994) have found either no relationship or an inverse relationship between total energy intake and colon cancer risk. On the other hand, case-control studies (Potter and McMichael, 1986; Whittemore et al., 1990; Peters et al., 1992) have found that cases reported greater caloric intake than controls suggesting an association between high energy intake and increased risk for CC. A case-control study by Slattery et al. (1997) found an increase in CC risk associated with high energy intake, but only at lower levels of lifetime vigorous physical activity and higher body mass index (BMI). These studies suggest it is possible that total energy intake has no simple relationship with colon cancer risk, but its effect maybe dependent on levels of obesity and physical activity.

Type 2 diabetes mellitus (DM) has emerged as another risk factor for CC. Limburg et al. (2005) examined the relationship between type 2 DM and CC risk in postmenopausal women participating in the Iowa Women's Health Study. They found that women having type 2 DM had a significantly increased risk of developing CC when compared to women with no history of DM (relative risk = 1.4). Cancer subsite-specific analysis found that type 2 DM increased the risk for proximal CC (relative risk = 1.9), but was not associated with increased risk for cancer in the distal colon (relative risk = 1.1) or the rectum (relative risk = 0.8). Coughlin et al. (2004) examined the relationship between DM and CC risk in a large cohort of men and women who had no history of cancer at enrollment in 1982. After 16 years of mortality follow-up, DM was significantly associated with CC in men (relative risk = 1.20) and women (relative risk = 1.24).

Other established non-dietary factors that influence CC risk include advanced age, low levels of physical activity, genetic pre-disposition, ulcerative colitis and smoking. Regular use of aspirin and/or NSAIDS has been associated with a decreased risk for colon cancer in humans (Waddell et al., 1989; Labayle et al., 1991).

1. Role of diet in etiology and prevention of colon carcinogenesis

1.1. Effect of dietary mutagens and carcinogenesis

Preventing dietary carcinogens and mutagens from interacting with the colon mucosa and inhibiting initiation of CC has been one of the many approaches employed in reducing diet induced CC. Carcinogens and mutagens include agents that occur in diet naturally or agents which are inadvertently added or produced during cooking or food

processing. *In vitro* mutagen assays such as the *Salmonella typhimurium* (Ames) test can identify potential genotoxic agents in the diet (Maron and Ames, 1983). When dose, threshold effects and *in vivo* detoxification mechanisms were considered, neither the naturally occurring nor inadvertently added dietary genotoxins accounted for the higher incidence of CC observed in Western countries compared to Poland, southern Europe, Japan or South America (Henderson, 1990). Potent mutagens such as polycyclic aromatic hydrocarbons, can be formed during charcoal broiling of meat (Lijinsky and Ross, 1967; Barnett, 1976). However, research by Weisburger (1973) and Toth (1980) have shown that polycyclic aromatic hydrocarbons do not significantly increase the risk for CC. Subsequent research focused on the formation of heterocyclic aromatic amines during frying, broiling and toasting of foods. These are considered extremely potent mutagens (Sugimura, 1992; Weisburger et al., 1994) and their presence in food may be one cause for the increased CC incidence in Western countries.

1.2. Reduction of contact between colon mucosa and carcinogens

An alternative approach in prevention of mutagenesis is to reduce contact between a mutagen/carcinogen and the colon mucosa. This can be achieved by diluting intestinal contents and/or by decreasing residence time of undigested food in the colon. Some types of dietary fiber can fulfill both roles. Non-fermented dietary fiber constituents like lignin or poorly-fermented dietary fiber constituents like cellulose and hemicellulose adsorb and absorb water. These fibers plus their associated water dilute intestinal contents and any carcinogen or mutagen that may be present. Also, dietary cellulose and hemicellulose decrease intestinal transit time through the colon. Pectins and

gums are often completely fermented by colonic microflora and, therefore, contribute little dilution of carcinogens and mutagens in the colon and do not decrease residence time of fecal material in the colon. Epidemiological studies suggest that dietary fiber is important in reducing CC incidence (Greenwald et al., 1987; Trock et al., 1990). However, results from animal studies that tested the efficacy of dietary fiber in reducing chemically-induced CC have been mixed – finding decreased, no effect, or even an enhancement of colon carcinogenesis (Glauert et al., 1981). Even though a preponderance of evidence suggests that dietary fiber decreases CC risk (Macquart-Moulin et al., 1986; Kune et al., 1987; Graham et al., 1988; Zaridze et al., 1993), the extent to which it can reduce CC incidence is uncertain. In contrast, recent comprehensive prospective studies examining the role of fiber and its components on risk of colorectal neoplasms have found no protective effect from a high-fiber diet against colorectal cancer or adenomas (Fuchs et al., 1999; Alberts et al., 2000; Bonithon-Kopp et al., 2000; Michaels et al., 2000; Schatzkin et al., 2000).

1.3. Role of colonic microflora

Differences in CC incidence between countries with high and low incidence rates have been hypothesized to result from differences in colonic bacterial populations or from differences in bacterial metabolites (Hill et al., 1971). Studies enumerating facultative and strict anaerobes in colon contents failed to find major differences in numbers or types of bacteria in the colons of persons at high versus low risk for CC (Moore and Holdeman, 1975; Goldberg et al., 1977). Although types or number of colonic bacteria were not associated with CC incidence, populations that have a high

incidence of CC have greater concentrations of mutagens in their stools (Reddy et al., 1978; Van Tasslee et al., 1990). Fecapentaenes are bacterial metabolites of unabsorbed fatty acids and they are mutagenic in bacterial mutation assays. However, fecapentaenes do not act as initiators of CC (Weisburger et al., 1990) as was originally hypothesized. A later study showed that fecapentaenes were more likely to function as promoters of CC (Zarkovic et al., 1993). Colonic concentrations of fecapentaenes are increased with high fat, low fiber diets. Therefore, it is possible that fecapentaenes contribute to the increased incidence of CC observed in populations consuming high fat, low fiber diets.

1.4. Prevention of cellular damage and antioxidants

Stimulating the activities of hepatic and intestinal "phase II" enzymes that detoxify xenobiotics can reduce chemically induced CC (Deschner et al., 1991; Yoshimi et al., 1992). Phase II enzymes conjugate xenobiotics with glutathione, glucuronic acid, sulfate, or acetate to xenobiotics to reduce their toxicity and to speed their clearance via bile or urine. Epidemiologic studies often find an inverse relationship between CC risk and consumption of fruits, vegetables and grains (Potter, 1996; Potter, 1999). This inverse relationship generally is attributed to increased consumption of antioxidants, fiber, and substances that enhance phase II enzymes (Steinmetz and Potter, 1991; Steinmetz and Potter, 1996). However, the results of the multi-center, prospective Polyp Prevention Trial found that consumption of a low fat, high fiber diet did not reduce polyp recurrence during a four-year intervention period (Schatzkin et al., 2000). More recent analysis of carotenoid and vitamin A consumption among a sub-cohort of the Polyp Prevention Trial indicated that higher serum concentrations and dietary intakes of α-

carotene and β-carotene, as well as higher dietary vitamin A consumption, all were associated with reduced risk for polyp recurrence (Steck-Scott et al., 2004). In another study comparing plasma antioxidant concentrations and polyp incidence in subjects undergoing complete colonoscopy, Erhardt et al. (2003) found that plasma lycopene concentrations were significantly lower (35%) in subjects having adenomas as compared to subjects without adenomas.

Oxidative damage to critical cellular macromolecules can both initiate and promote CC (Cerutti and Trump, 1981; Wattenberg, 1993; Lippman et al., 1994). Carotenoids, vitamins C and E, phenolics and selenium, as part of the glutathione peroxidase enzyme complex, act as general antioxidants at the cellular level. However, it is difficult to show that dietary intakes above the minimum requirements offer a protective effect (Greenberg et al., 1994; MacLennan et al., 1995; Clark et al., 1996).

Inflammatory conditions stimulate oxidative damage to cells, production of growth factors and cell proliferation leading to promotion of CC (Cerutti and Trump, 1981; Baldassarre et al., 2004).The prostanoids - prostaglandin E1, prostaglandin E2 and leukotriene B4 - are potent proinflammatory agents. Inhibition of prostanoid synthesis can reduce the oxidative damage to cells and tissue and thus inhibit CC. Non steroidal anti-inflammatory drugs (NSAIDS) that inhibit prostanoid synthesis also lead to regression of colon polyps in humans (Earnest et al., 1992; Giardiello et al., 1993; Reddy et al., 1993; Pennisi, 1998; Baron et al., 2002) and inhibit chemically induced CC in rodent models (Labayle et al., 1991; Reddy et al., 1996; Kawamori et al., 1998).

1.5. Fat consumption and caloric intake

Dietary fats, particularly the saturated fat present in red meat, may contribute to colorectal cancer (Willett et al., 1990). Multiple case-control studies also have demonstrated an association between high fat consumption and increased CC risk (Potter, 1992; Giovannucci et al., 1994). Animal studies suggest that high fat intake, particularly saturated fat, induces abnormal proliferation of colonic mucosa and may even lead to development of aberrant crypt foci, one of the earliest cancer-associated structural changes in the gut (Newmark et al., 1991). Bile acids whose production is increased by high fat intake have mitogenic effects upon intestinal epithelial cells (Hill et al., 1971; Chomchai et al., 1974). Secondary bile acids (deoxycholic and lithocholic acid) are present in higher concentrations in the colon of populations at increased risk for CC. Because this population also had an increased fat and decreased fiber diet, the exact relationship of these components to CC risk remains uncertain.

There has been considerable interest in the relationship between calorie intake and colon carcinogenesis. When investigating their relationship with any chronic disease it is difficult to distinguish between the energy intake and intake of dietary fat. Thus the question has been raised whether the effect of dietary fat on colon carcinogenesis is due to specific action of fat or to an associated caloric effect (Carrol, 1986; Reddy, 1986). Several studies have reported that calorie restriction reduces the formation of spontaneous and chemically induced tumors in mice (Ross and Bras, 1971). Further studies have shown that calorie restriction decreased oxidative damage to DNA (Klurfeld et al., 1987). The effect of a high-fat, semi-purified diet injested ad libitum and of a 30% calorie restricted diet on AOM-induced colon carcinogenesis during the post initiation

phase was investigated by Reddy et al. (1987). The animals on the calorie- restricted diet developed significantly fewer colon tumors and had a lower colon tumor incidence than the rats in the ad libitum-fed group. In another study (Kumar et al., 1990) the effect of 10%, 20%, and 30% calorie restriction was investigated and it was found that the incidence and multiplicity of colon tumors were significantly inhibited in animals fed 20% and 30% calorie-restricted diets. These results indicate calorie restriction elicits a dose response effect on inhibition of tumor incidence.

2. Diet and Chemoprevention of colon cancer

2.1. Chemoprevention

The epidemiology of colorectal cancer suggests that the most direct method of preventing colorectal cancers is by identification and removal of precursor adenomas. However, the multi-step nature of colonic neoplasia makes early detection difficult (Loren et al., 2003). In advanced stages of the cancer, surgical resection of the colon and adjuvant chemotherapy or radiotherapy is practiced. Because reduced risk of colorectal neoplasia is associated with increased dietary intakes of various chemicals with cancer-preventing properties in animal studies, an intense effort is underway to develop effective and safe chemopreventive agents (Hawk et al., 2004). An ideal chemopreventive agent would be simple to administer with extremely low toxicity, as continuous exposure may be necessary for prevention of colon cancer (Courtney et al., 2004).

A number of promising chemopreventive agents such as dietary calcium, soy phytochemicals, citrus bioflavanoids, cherry anthocyanins, COX 2 inhibitors (NSAIDS), and others have been identified which modulate cellular processes such as cellular

proliferation, differentiation and apoptosis, and thereby may have efficacy in reducing CC risk (Hawk et al., 2004). The following sections describe the potential of soy phytochemicals and calcium to reduce CC risk

2.2. Soy consumption and colon cancer

i. Epidemiological studies

International variations in colon cancer have been attributed, at least in part, to differences in dietary intake. Asian populations have relatively low rates of breast, prostate, and colon cancer when compared with Western cultures. Studies of migrant populations have helped confirm the hypothesis that environmental variables (especially diet) play a significant role in determining cancer risk. For example, rates of breast cancer are lower in Japan than in the United States and increase among Japanese migrating to Hawaii (Parkin, 1989). Asian diets are typically lower in total and saturated fat and higher in dietary fiber than Western diets. Another significant dietary difference between Asian and Western population is the consumption of soy-containing foods. Soybeans have long been a major component of the Asian diet, common soy products being soymilk, tofu, miso, yuba and tempeh. Mean intakes of 20-50 grams of soy foods per day, has been reported in studies, but the values for soy intake on dry weight basis is much lower (Wu et al., 1998).

In a review of epidemiologic literature, Messina et al. (1994), examined the relationship between consumption of soy-containing foods and cancer risk. Among twenty-five studies of fermented soy products reviewed, four noted an increased risk, eighteen no statistically significant difference and three found a decreased risk of cancer

development of various sites (breast, prostate, colon, lung). Among twenty-six studies of non-fermented soy products, results were more consistent in showing either a decreased risk (10 studies) or no statistically significant difference (15 studies), with only one study showing an increased risk of esophageal cancer associated with consumption of fried bean curd.

In a cross-cultural study of thirty-eight countries, McKeown-Eyseen and Bright-See (1984) found no association between soybean intake and CC risk. The countries studied were chosen on the basis of reliable cancer mortality data but were not identified. Therefore, it is difficult to evaluate findings from this study since only a handful of countries consume amounts of soy likely to bear any physiological relevance. Haenszel et al. (1973) reported a significantly increased risk of CC among Japanese born Issai residing in Hawaii who ingested fermented soybeans (relative risk = 1.6). Tajima and Tominaga (1985) in a Japanese study, noted an increase in rectal, but not in CC risk, in persons ingesting miso soup (relative risk = 2.05, $P<0.05$). These authors found no relationship between bean curd consumption and either colon or rectal cancer.

Three other case-control studies have been suggestive of a protective effect of soy consumption although differences were statistically significant in only one of these studies. Poole et al. (1989) in the only case-control study conducted in the United States, noted a relative risk of 0.53 (confidence interval 0.11-2.66) for CC for persons eating tofu/soybeans more than once a month, as opposed to less than once a month. Hu et al. (1991) found a protective effect of soybean products on rectal, but not on colon, cancer risk in a case-control study conducted in China. Watanabe et al. (1984) found that consumption of beans and bean curd once or twice a week and three or more times a

week, in comparison to not consuming theses foods, was associated with lowered rectal cancer risk (relative risks = 0.15 and 0.12 respectively). They found a lower, but not significantly decreased risk of CC (relative risk = 0.63).

More detailed discussion of these studies can be found in a (Messina and Bennink, 1998) review of 8 ecological and case-control studies that examine the relationship between soy food intake and colorectal cancer. None of these studies reported a statistically significant reduction in CC risk with soy consumption. They observe that while these studies report a non-statistical reduction in colon cancer risk, there were a few that associate certain soy product consumption with a higher risk for CC. However, in most of these studies, soy intake and cancer risk was not the primary focus of investigation, and there is cause for concern with regards to the completeness and accuracy of the dietary soy data in these studies. Spector et al. (2003) in her review of 13 epidemiological studies (3 ecological, 1 cohort and 9 case-control) observed that these studies were inconclusive with only a suggestion of possible inverse association between soy products and colorectal cancer. Therefore, it is safe to conclude, while there is no compelling epidemiologic evidence to support an inverse relationship between soy intake and risk of colorectal cancer, there exist a need for studies that are designed specifically to determine if soy consumption lowers the risk for colon cancer.

ii. Animal studies

Much research has been done regarding the effect of feeding isolated soy protein and soy isoflavones on the development of ACF in rats given a chemical carcinogen. At least three studies showed that soy diets or isoflavones inhibited the formation of ACF

(Pereira et al., 1994; Helms and Gallaher, 1995; Masaoka et al., 1998). A dose-dependent inhibitory effect was also reported in these studies (Pereira et al., 1994; Helms and Gallaher, 1995; Masaoka et al., 1998). Two other studies failed to show an inhibition of ACF formation by isoflavone supplement. In a study reported by Davies et al. (1999), isoflavones (570 mg/kg diet) did not inhibit formation of ACF when compared to a casein-based control diet. Gee et al. (2000) performed two experiments with genistein or soy protein isolate given at different time points relative to carcinogen administration. Feeding genistein or soy protein isolate prior to the injection of dimethyl hydrazine (DMH) increased the formation of ACF by 2-3 fold, while there was no effect of genistein or soy protein isolate when administered after the treatment of DMH.

Most animal experiments have not shown a significant protective effect of soybean products given as a source of protein in chemically-induced colorectal tumorigenesis (Clinton et al., 1979; McIntosh et al., 1995). The incidence of colorectal tumors induced by DMH was not lower in rats fed soy protein than those fed meat protein (McIntosh et al., 1995). An isoflavone rich diet (500-600mg /kg diet) did not inhibit tumorigenesis in either AOM-treated rats or the Min mouse model (Sorensen et al., 1998; Davies et al., 1999). The Min mouse has single mutated copy of the APC gene and is a model for human familial adenomatous polyposis. However, a recent study, found that soy protein isolate containing 430mg of isoflavones per kg diet substantially decreased AOM-induced colorectal tumors in the F2 generation of rats, with their parents fed the same diet before mating (Hakkak et al., 2001).

iii. Chemical composition of soy and proposed anti-carcinogenic compounds in soy

The composition of soybeans may vary somewhat according to variety and growing conditions. The average proximate composition of soybeans is 40% protein, 20% lipid, 35% carbohydrates and 4.9% ash. Through plant breeding it has been possible to obtain protein levels ranging between 40% and 45%, and lipid levels between 18% and 20%. Several putative anti-carcinogenic phyto-chemicals have been identified in soybeans. These include isoflavones, protease inhibitors, phenolic acids, phytosterols, phytates, and saponins.

The variety of soy products available for human consumption and used for research differ markedly in their concentrations of macronutrients (protein, fat, and carbohydrates) and micro-constituents (isoflavones, saponins, phytic acid, phytosterols, vitamins, and minerals; Endres, 2001). Methods of processing can dramatically influence the concentrations of isoflavones and other phytochemicals present in soy products (Figure 1). De-fatted soy flour (obtained by hexane extraction of soy flakes) contains significant concentrations of isoflavones and other phytochemicals. However, ethanol extraction of soy flour to produce soy protein concentrate removes the majority of the isoflavones and oligosaccharides that were originally present in soy flour (U.S. Department of Agriculture, 1999). Table 1 shows the substantial compositional differences in macronutrient content among soy flours, concentrates, and isolates. The chemical composition of soy concentrates or isolates is affected by processing parameters such as solubilization method and temperature. Either water or alcohol (ethanol) washing can be used to concentrate the protein fraction by removing sugars and oligosaccharides from soy. Water and, to some degree, ethanol will also remove some of the low-

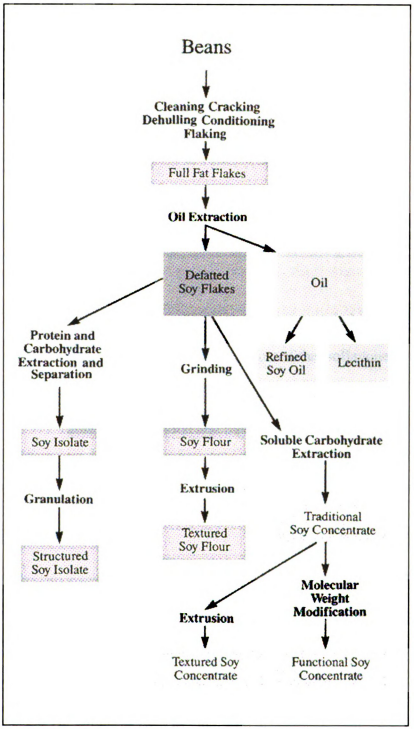

Figure 1. Stages of soy bean processing

Table 1. Percentage composition of soy protein products (moisture-free basis)[1]

Constituent	Defatted flours and grits	Concentrates	Isolates
Protein (N x 6.25)	56-59	65-72	90-92
Fat	0.5-1.1	0.5-1.0	0.5-1.0
Crude fiber	2.7-3.8	3.5-5.0	0.1-0.2
Soluble fiber	2.1-2.2	2.1-5.9	<0.2
Insoluble fiber	17-17.6	13.5-20.2	<0.2
Ash	5.4-6.5	4.0-6.5	4.0-5.0
Carbohydrates (by difference)	32-34	20-22	3-4

[1] Modified from Endres, 2001.

molecular-weight peptides (those below 2000 daltons) that contain high amounts of sulfur amino acids. Alcohol washing also removes most of the isoflavones (and other lipid-soluble phytochemicals such as saponins) from the product (Coward et al., 1993; Murphy et al., 1999). Table 2 lists the total and individual isoflavone contents of selective soy foods (Jackson and Gilani, 2002).

Researchers often compare effects of ethanol-washed with un-washed soy protein and assume that any differences observed in health outcomes are due to the isoflavone component. However, saponins, other alcohol soluble constituents, and the protein and peptide composition could also contribute to disparate health effects of these soy preparations. The composition of commercialized soy foods varies substantially. Setchell and Cole (2003) recently evaluated the variations in isoflavone levels in soy foods and soy protein isolates. They found a two- to three-fold variation in total isoflavones in soy protein isolates obtained over three years and five-fold differences in isoflavone levels in different commercial soy milks. The differences among products are due in part to the variety of soybeans, differences in growing and storage conditions, and differential food

Table 2. Estimated isoflavone contents of selected soy protein products[1,2]

Description	Daidzein	Genistein	Glycitein	Total isoflavones
Soy flour, full-fat, roasted	99.27	98.75	16.40	198.95
Soybean flakes defatted	36.97	85.69	14.23	125.82
Soy protein concentrate, aqueous washed	43.04	55.59	5.16	102.07
Soy protein concentrate, produced by alcohol extraction	6.83	5.33	1.57	12.47
Soy protein isolate	33.59	59.62	9.47	97.43

[1] Modified from Jackson and Gilani, 2002

[2] Values based on mg/100 g edible portion

processing techniques. Therefore, it is important to accurately assess the chemical composition and phytochemical contents of soy products when investigating the potential health effects of these products. The following sections will review evidence on the potential efficacy of these compounds to influence CC risk.

a). Isoflavones

Isoflavones are a group of naturally occurring heterocyclic phenols found in soybeans and forage plants. Genistein (4', 5, 7-dihydroxy isoflavone), and daidzein (4', 7-dihydroxy isoflavone), occurring as their beta-glucosides genistin and daidzin, respectively are the principal isoflavones found in soybeans consisting as much as 90% of the total isoflavone content (Wang and Murphy, 1994b). Soybeans are a particularly unique and rich source of isoflavones in the human diet, containing genistein and daidzein at levels up to 3 mg/g of dry matter (Coward et al., 1993). Genistein and daidzein derivatives usually are present in roughly equal concentrations in soybeans and soy products. Isoflavones are insoluble in solvents typically used for soybean oil

extraction. Hence, soy flour (50% protein) is a rich source of isoflavones, containing about 3 mg of total isoflavones per gram of dry matter. Other Asian style soy-based products (soy milk, tofu, soy powder and soy nuts) have total isoflavone concentrations in the range of 1.3 - 3.8 mg/g dry weight. Extraction of soy flour with alcohol to produce soy concentrate (65% protein) results in low concentrations of isoflavones in the concentrate as isoflavones are soluble in alcohol. Soy concentrates prepared by water extraction contain isoflavone concentrations similar to that found in soy flour. A portion of isoflavones are lost during preparation of soy protein isolate (90% protein). Soy protein isolates prepared by conventional means (alkaline solubilization following acid precipitation of protein) contain approximately 1 mg or more total isoflavones per gram dry matter. Soy fiber contains only small concentrations of isoflavones as they are normally associated with the protein fraction of the soybean.

Soy products that are prepared by processes that do not involve fermentation normally contain isoflavones in their conjugated forms. However, fermentation of soy products results in the hydrolysis of isoflavone glycosides, releasing their aglycone forms. Tempeh and miso, which are fermented soy products typically consumed in Asian countries, contain a significant quantity of their total isoflavones in the aglycone form. Non-fermented soy-foods typically consumed in Asia, such as soy milk and tofu, primarily contain isoflavone glycosides. Soy sauce contains only a minor concentration of isoflavone glycosides (Coward et al., 1993; Wang and Murphy, 1994a; Franke and Custer, 1996).

b). Nutritional intake

The issue of what constitutes normal dietary levels of isoflavone intake, particularly in Asian populations where soy foods are a staple, is controversial. Based on urinary isoflavone excretion, researchers first suggested that typical intakes of isoflavones ranged from 50 to 150 mg/day in Japanese adults (Setchell et al., 1984). This was later disputed and a more conservative estimate of 50 mg/day was proposed as more likely (Messina, 1995). There have been no direct attempts to estimate daily intake of phytoestrogens in Japanese adults, although several have used dietary recall to provide estimates of soy food intake in persons living in Japan (Wilcox et al., 1995; Nagata et al., 1998; Wakai et al., 1999), China (Chen et al., 1999), Indonesia (Purba et al., 1999), and Japanese-Americans in Hawaii and Canada (Wilcox et al., 1995; Maskarinec et al., 1998; Nagata et al., 1998; Chen et al., 1999; Purba et al., 1999; Wakai et al., 1999). Nagata et al. (1998) reported that the average daily amount of soy foods consumed by Japanese adults is 54.4 and 63.6 g for women and men, respectively. However, it should be noted that there was a huge individual variation. This intake of soy foods corresponds to 8.00 g and 6.88 g of total soy protein for women and men, respectively. From these figures, a reasonable assessment of isoflavone intake can be calculated by assuming 2–5 mg isoflavones per gram soy protein (Setchell and Cassidy, 1999). Based on this assumption, Japanese adults probably consume 15–45 mg of isoflavone/day on average. Estimates of isoflavone intakes by Chinese adults are similar (Chen et al., 1999), while it is evident that Indonesians must ingest much higher levels based on the relatively large quantities of tofu and tempe consumed (Purba et al., 1999). Recent published figures show the median intakes of these foods to be 125 g/day for elderly people living in Jakarta with the range

being 62 and 250 g/day for the 25th and 75th percentiles of intake. This represents a considerable intake of soy foods and while there is a wide variability in isoflavone content of tempe and tofu, this study would suggest that intakes in excess of 150 mg/day may be attainable. Interestingly, the incidence of breast and prostate cancers in Indonesia are considerably lower than observed in Japan and China, and the incidences of these cancers would appear to be inversely correlated with the isoflavone intakes observed in these three countries. Intakes of isoflavones by Asians are much higher than those of Westerners, which are clearly negligible based upon the extremely low isoflavone concentrations found in urine and plasma. Other studies on isoflavone intakes in Asian countries and in Asian populations in Western countries seem to support these low estimates of the Westerners (Yuan et al., 1995; Wu et al., 1996; Wu et al., 1998; Chen et al., 1999).

c). Overview of metabolism

The biologic activity and metabolic fate of dietary soy isoflavones differ depending on their chemical form (Kelly et al., 1993; Cassidy et al., 1994; Xu et al., 1994; Joannou et al., 1995; Knight and Eden, 1995; Cassidy, 1996). Whereas structure itself is not a limiting factor for absorption from the gastrointestinal tract, (Hendrich et al., 1999) the chemical form of the isoflavone and its metabolites influence the extent of absorption, with aglycones more readily absorbed and more bioavailable than highly polar conjugated species (Setchell et al., 1984; Izumi et al., 2000; Setchell, 2000).

Following ingestion, the acetyl and malonyl derivatives of genistin and daidzin are metabolized to genistin and daidzin, which are then hydrolyzed in the large intestine

by bacteria, resulting in the removal of the sugar moiety to produce their respective aglycones, daidzein, and genistein (Setchell et al., 1984; Kelly et al., 1993; Izumi et al., 2000; Setchell, 2000). Following absorption of the aglycones, these compounds and their metabolites are readily conjugated in the liver with glucuronic acid and/or sulfate, circulate enterohepatically with potential metabolism and reabsorption in the intestine (Barnes et al., 1996). Those isoflavones that are not absorbed are excreted in the unconjugated form in feces (Adlercreutz et al., 1995). The glucuronide fraction, the predominant conjugate representing up to 90% of circulating isoflavones in both rats and humans, (Holder et al., 1999; Doerge et al., 2000) is considered biologically inactive, (Cassidy, 1996) whereas the free and sulfated fractions, present at much lower concentrations, are generally thought to be biologically active. Alternatively, daidzein may be further metabolized by resident microflora in the gastrointestinal tract to equol and O-desmethylangolensin (via their respective intermediates, dehydroequol and dihydrodaidzein) (Kelly et al., 1993; Bayer et al., 2001). Similarly, genistein may be metabolized to 6'-hydroxy-O-desmethylangolensin via the intermediate dihydrogenistein (Joannou et al., 1995) and further to p-ethyl-phenol (Goldwyn et al., 2000). A recent *in vitro* study by Kulling et al. (2000) identified hydroxylated metabolites of both genistein and daidzein using liver microsomes from Aroclor-treated male Wistar rats, which the authors suggested might function as an important metabolic pathway *in vivo*. Metabolites of glycitein have recently been tentatively identified in human urine as 5'-OH-O-desmethylangolensin and 5'-methoxy-O-desmethylangolensin (Heinonen et al., 2000).

The absorption, distribution, metabolism, and excretion (ADME) of soy isoflavones vary, based on age or gender, and among cultural groups. Interindividual

variability has been documented in several studies, (Kelly et al., 1995; Xu et al., 1995; Gooderham et al., 1996; Morton et al., 1997; Xu et al., 2000) although evidence that isoflavones may induce their own metabolism in some individuals is inconclusive (Lu et al., 1995; Barnes et al., 1996; Lu et al., 1996; Karr et al., 1997). Investigation of variability in the ADME of isoflavones between males and females has produced inconsistent results (Lockhart et al., 1978; Kirkman et al., 1995). Absorption and excretion of isoflavones have been reported in infants, with evidence that these may vary because the underdeveloped gut microflora cannot hydrolyze the glucuronide forms (Cruz et al., 1994; Huggett et al., 1997). Additionally, differences in metabolic pathways may arise owing to different subpopulations of microflora, intestinal transit time, pH, or redox potential, (Hendrich et al., 1999) factors that are influenced by diet, drugs (including antibiotics), bowel disease, surgery, and host immunity (Knight and Eden, 1995). Differences among cultural or geographic populations have not been systematically investigated, although it has been reported that some individuals are not able to form equol (Adlercreutz et al., 1991; Adlercreutz et al., 1995; Barnes et al., 1996).

Pharmacokinetic studies demonstrate that after oral ingestion of individual isoflavones, peak plasma concentrations are attained 5–6 hours later for the aglycones and the clearance from plasma proceeds with a half-life of systemic elimination of 6–8 hours (Setchell et al., 2001). Notable differences are seen in the pharmacokinetics of daidzein and genistein and their corresponding β-glycosides. Plasma concentrations of genistein are consistently higher than daidzein when equimolar amounts are ingested. This is attributed to the much greater volume of distribution of daidzein compared with genistein and its higher clearance rate. The bioavailability of genistein is higher than that

of daidzein and the overall bioavailability of isoflavones is highest when they are ingested as aglycones versus β -glycosides, as determined following single-bolus oral administration (Setchell et al., 2001). The rate of absorption of the aglycones is much faster than that of the β -glycosides (Setchell et al., 2001), a finding also confirmed in another study comparing a fermented and unfermented soy food product (Izumi et al., 2000). This would be predicted based on chemical structure, because at normal intestinal pH the aglycones will be rapidly absorbed by a process of non-ionic passive diffusion. Setchell et al. (2001) demonstrated that the time required to reach the maximal plasma concentration of isoflavones is significantly longer when the β-glycosides are ingested. However, the aglycones are more vulnerable than the corresponding β -glycosides to further degradation to an array of other metabolites (Kelly et al., 1993; Joannou et al., 1995), thus limiting their bioavailability. Setchell also revealed a curvilinear relationship between the systemic bioavailability (as measured from the AUC of the plasma concentration curves) and amount of isoflavones ingested, at least where food is concerned (Setchell, 2000). Based on the pharmacokinetics, maximum steady-state plasma levels are more likely to be attained by repeated ingestion throughout the day of several servings of soy foods having modest isoflavones levels. This contention is supported by data from studies of infants fed soy formulas where the plasma levels attained by repeated feeding throughout the day are approximately tenfold higher than those observed in adults consuming similar quantities of isoflavones (Setchell et al., 1997).

d). Biological effects

In vitro studies have indicated that isoflavones have a number of interesting biological effects. Genistein is well known as a weak estrogen receptor agonist with an activity that is approximately $1/1000^{th}$ that of estradiol. Genistein has been demonstrated to inhibit the activities of a number of cellular enzymes involved in cell cycle regulation, including both receptor and cytosolic protein tyrosine kinases (Akiyama et al., 1987; Makishima et al., 1991), ribosomal S6 kinase (Linassier et al., 1990), MAP kinases (Thorburn and Thorburn, 1994), DNA topoisomerases I and II (Constantinou et al., 1990), epidermal growth factor induced phosphatidyl inositol turn over (Imoto et al., 1988) and increase transforming growth factor β (TGF β; Peterson and Barnes, 1996). Genistein also has been demonstrated to elicit anti-oxidant (Jha et al., 1985; Wei et al., 1995), anti-proliferative (Yanagihara et al., 1993), and anti-angiogenic (Fotsis et al., 1993; Raines and Ross, 1995) activities. Daidzein is not an effective inhibitor of tyrosine kinases, but has been found to inhibit the activity of casein kinase II (Higashi and Ogawara, 1994).

Most of the anti-carcinogenic potential of isoflavones has been inferred from effects of isoflavone aglycones on the growth of cancer cells in culture. Several *in vitro* studies have shown that genistein inhibits growth of a wide range of both hormone-dependent and hormone-independent cancer cell lines with IC_{50} values ranging from 5–100 μM (approximately 2–25 μg/ml). In addition, genistein administration has been shown to inhibit the *in vivo* metastatic activity of both breast (Scholar and Toewa, 1994) and prostate cancer cells (Santibanez et al., 1997) independent of effects on cell growth. Genistein inhibits the growth of colon cancer cell lines HCT-8 (Clark et al., 1989) HCC-

48 and HCC-50 (Yanagihara et al., 1993), and caco-2 and HT-29 (Kuo, 1996) in a dose dependent manner.

The precise mechanisms by which genistein inhibits growth of cancer cells are still unknown, but several potential mechanisms have been proposed. Inhibition of cell growth induced by genistein is associated with cell cycle arrest at $G_2 - M$ and induction of differentiation and apoptosis (Matsukawa et al., 1993; Pagliacci et al., 1994; Shao et al., 1998). The effects of genistein on cell growth are believed to be mediated largely by the inhibition of key enzymes (tyrosine kinases, MAP kinases, DNA topo-isomerases) involved in cell cycle regulation. Enzymes in the protein tyrosine kinase family phosphorylate the tyrosine residues on key proteins involved in signal transduction events in normal and tumor cells (Akiyama et al., 1987; Makishima et al., 1991). Gensitein inhibits DNA topoisomerase I and II, which are involved in DNA replication. Peterson and Barnes (1996) reported that genistein increased the *in vitro* concentrations of TGF-β and this may be important given the role of TGF-β in inhibiting the growth of cancer cells. In Caco-2 cells, genistein induced differentiation as measured by changes in alkaline phosphates and dipeptidyl peptidase, and this enzyme induction was correlated with the phosphorylation of three different proteins (Basson et al., 1998). These results are consistent with earlier studies that showed genistein inhibited gastrin-stimulated growth in non transformed intestinal crypt cells (Raid et al., 1993) and in colon mucosa (Majumdar, 1990). The blockade in gastrin stimulation of cell growth by genistein was mediated by reduced tyrosine phosphorylation of several proteins (Majumdar, 1990; Raid et al., 1993).

In vitro studies indicate that the inhibitory effect of genistein on cancer development can only be achieved at relatively high concentrations. The IC_{50} required for growth inhibition is typically greater than 13.2 μmol genistein/L (Barnes et al., 1995) and can vary as high as 90 μmol genistein/L (Kuo, 1996). However, plasma levels of genistein in people consuming soy rich foods have estimated to be in the range of 1–6 μmol genistein/L (Xu et al., 1995; King and Bursill, 1998; Adlercreutz et al., 2000). Given the short half- life of genistein (4.8–5.7 hours), and the fact that most of plasma genistein is conjugated with glucuronic acid, a very high intake of and a repeated exposure to genistein is required to achieve plasma concentrations in the range of *in vitro* concentrations required to inhibit cancer cell growth.

Various *in vitro* and *in vivo* assays show genistein to be a weak estrogen receptor agonist, exerting an estrogenic effect ranging from approximately 1×10^{-3} to 1×10^{-5} that of 17-β estradiol (Mayr et al., 1992; Markiewicz et al., 1993). Weak estrogens have been postulated to function as anti-estrogens *in vivo* (Martin et al., 1978) and thus, theoretically, may reduce the risk of hormone-dependent cancers such as breast and prostate cancer (Setchell et al., 1984). However, this effect seems to be tissue specific and in tissues such as heart and bones genistein has an estrogenic effect. Traditionally, CC has not been regarded as being a hormone-dependent cancer, but some evidence suggests that CC can be hormonally influenced. Although world wide CC incidence rates among men and women are similar, there is a gender variation in the sub sites of CC. McMichael and Potter (1980) have reported a female excess of right-sided colon cancers and a male excess of left-sided cancers. This may be due to differences in gut metabolism, colonic bacterial populations, and fermentation rates between the sexes, which could be mediated

ultimately by hormones. Studies have also shown that there was a brief decline in CC incidence among women compared with men in countries where use of the original high-dose oral contraceptives were common. McMichael and Potter (1980) also suggested that oral contraceptive use was associated with low rates of CC in women. Chen et al. (1998) found that women who used hormone replacement therapy (HRT) in the year prior to sigmoidoscopy, were only half as likely to have colorectal adenomas compared to the women who did not use HRT. Similarly, in a case-control study conducted by Newcomb and Storer (1995) it was found that recent users of HRT had a CC relative risk of 0.54 when compared with post-menopausal women who never used HRT. Grodstein et al. (1998), in a prospective study involving 59, 000 post-menopausal women, found that current users of HRT had a relative risk for colorectal cancer of 0.65. In contrast, Maclennan et al. (1995) had published a meta-analysis on fourteen studies related to HRT and colon cancer that concluded that HRT was not protective. As soy isoflavones have been shown to exert both estrogenic and anti-estrogenic effects, they may have the potential to affect CC risk through hormonal mechanisms, if a role for estrogen is established in the etiology of CC.

As mentioned previously, some research confirms a protective effect of isoflavones against carcinogenesis in animal models. Pereira et al. (1994) tested the ability of genistein to inhibit the formation of ACF in colons of rats treated with 15mg/kg body weight AOM and found that the addition of 75 and 150 mg genistein per kg of semi-purified diets fed to rats resulted in 29 % and 34 % reductions in ACF respectively. Although this study show that genistein inhibits pre cancerous lesions in the colons of rats, there are none that show if inhibition of ACF development would translate to a

reduction in colon tumors. In a related study by Bennink and Om (1998), animals fed defatted soy flour (total isoflavones 873 mg/Kg) resulted in a 47-49 % reduction in AOM-induced colon tumor incidence and a 60-74 % reduction in tumor burden in comparison to rats fed soy protein concentrate or casein. Feeding a mixture of genistin, daidzin and glycitin isoflavones (concentrations equivalent to those found in de-fatted soy flour) added in the soy concentrate diet did not influence tumor incidence but feeding genistin (equivalent to 500 mg/kg genistein) added to the soy protein concentrate increased tumor incidence (P = 0.06) (Bennink and Om, 1998). This result confirms the study by Rao et al. (1997) who found increased AOM-induced colon carcinogenesis in male F344 rats when fed diets containing 500mg genistein /kg diet beginning two weeks prior to, during and after carcinogen administration. However, it should be noted that in the study by Bennink and Om (1998) adding genistin or an isoflavone mixture to soy concentrate or feeding defatted soy decreased average tumor weight by about 50 %. These results suggest that genistin alone is sufficient to inhibit tumor growth but not tumor incidence. From the same study, it can also be concluded that defatted soy flour (with ethanol soluble phytochemicals) inhibited colon carcinogenesis. Therefore it appears that other ethanol soluble anti-cancer constituents such as, saponins, phytosterols, protease inhibitors and phenols that are present in the defatted soy flour may be responsible for the anti-cancer effect of de-fatted soy flour.

e). Protease inhibitors

Protease inhibitors are proteins found in cereals, nuts, fruits, vegetables, eggs, and potatoes with typical molecular weights between 8,000 and 10,000 daltons. As much as 6

% of all soy protein consists of protease inhibitors, making soy a particularly plentiful source of these compounds (Birk, 1990). Troll et al. (1980) initially proposed that soybean protease inhibitors could suppress cancer. Subsequent research determined that chymotrypsin-specific inhibitors, but not trypsin-specific inhibitors, suppress transformation *in vitro* (Kennedy, 1993). Feeding the soybean Bowman-Birk inhibitor (BBI, chymotrypsin inhibitor) suppressed chemically induced CC in mice (Weed et al., 1985; St. Clair et al., 1990).

The exact mechanism whereby chymotrypsin inhibitors can prevent cancer is not yet known. *In vitro* research demonstrated that BBI acts between the initiation and transformation stages of carcinogenesis. BBI does not prevent initiation, but it does irreversibly inhibit critical steps that lead to cell transformation. If a cell is already transformed, BBI cannot reverse transformation.

BBI has been evaluated as purified BBI (PBBI) or as an extract of soybeans enriched in BBI, termed BBI concentrate (BBIC), which is under evaluation in human trials as an anti-carcinogenic and anti-inflammatory agent. PBBI and BBIC have been shown to inhibit radiation- and chemically- induced malignant transformation *in vitro* (Kennedy, 1993) and animal carcinogenesis in DMH induced colon and liver carcinogenesis in mice (Weed et al., 1985; Billings et al., 1990; St. Clair et al., 1990) and spontaneous colon carcinogenesis in mice that are genetically susceptible to the induction of intestinal carcinogenesis (Kennedy et al., 1996). When Kennedy et al. (2002) fed soybean extract containing 0.5 % BBIC to Min mice, there was a 40% reduction in the number of tumors/mouse in the small intestine and 42% reduction in tumorigenesis in the colon (Lichtenstein et al., 2002). Billings et al. (1990) also reported a 45% reduction in

DMH induced colon adenocarcinomas with a diet containing 0.1% BBIC in rats. When animals are given dietary BBIC, it is known that BBI reaches the colon in an active firm (Owen and Jones, 1974) and enters colonic epithelial cells (Locniskar et al., 1985). The doses of 0.1% and 0.5% BBIC used in the animal studies are comparable to doses that are being studied in human cancer prevention trials (25-400 C.I. units) (Bland and Britton, 1975).

Additional studies regarding soy as a source of protease inhibitors to help prevent cancer is warranted. The anti-cancer activity of soy protease inhibitors is ascribed to the BBI, but the BBI in soy products is reportedly less effective than purified BBI or BBI concentrates (Kennedy, 1993). BBI is heat sensitive as protease inhibitor activity is destroyed by autoclaving and autoclaved BBI concentrate is ineffective in preventing oral carcinogenesis (Messadi et al., 1986). Therefore, how much BBI activity remains in heat processed soy foods (i.e., roasted soy nuts, spray dried soy protein isolate, or baked and cooked products containing soy protein) is not known. The quantity of BBI in soy concentrates is dependent upon the technique used to produce the concentrate. Concentrates prepared by aqueous alcohol extraction will have greatly reduced BBI activity since acidic 60 % ethanol is used to extract BBI in the preparation of BBI concentrate (Yavelow et al., 1985). Much research will be required to answer questions regarding the efficacy and safety of protease inhibitors as anti-carcinogens. Identifying soy products which can deliver biologically active BBI to humans is also needed.

f). Phytates

Graf and Eaton (1985) proposed that phytic acid (inositol hexaphosphate, IP_6), could decrease the incidence of CC. Since then, data from epidemiologic (Graf and Eaton, 1993), animal and cell culture studies (reviewed by Shamsuddin, 1995; Shamsuddin et al., 1997) have been published to support this hypothesis.

Several research groups (Pretlow et al., 1992a; Shamsuddin, 1995; Alabaster et al., 1996) have demonstrated that IP_6 can inhibit chemically-induced CC in animal models and that IP_6 can inhibit both the initiation and promotion stages of carcinogenesis. Both *in vitro* and *in vivo* studies suggest that IP_6 acts by inducing cell differentiation (Shamsuddin et al., 1997) or by reducing cell proliferation (Corpet et al., 1997). It is reported that IP_6 blocks activation of phosphatidyl inositol–3 kinase which in turn inhibits cell transformation and may account for reduced cell proliferation (Corpet et al., 1997) and enhanced cell differentiation (Shamsuddin et al., 1997).

There is very little doubt that isolated phytate fed at 1–3% of the diet can inhibit CC in animal models (Shamsuddin, 1995). However, phytic acid in plant material is mostly bound and exists as either protein-phytic acid complexes or as protein-phytic acid-cation complexes (Szwergold et al., 1987). Since complexed IP_6 is less available, it is less effective as an anti-carcinogen than isolated, neutralized phytate (Shamsuddin et al., 1997). Thus, it is unlikely that the potential of dietary soy to inhibit colon carcinogenesis is explained by its phytic acid content.

g). Phenolic acids

Some phenolic acids have anti-mutagenic activity *in vitro* and anti-carcinogenic activity *in vivo*. Defatted soy flakes contain about 1.1 mg of total phenolic acids per gram and soy isolate contains about 0.5 mg phenolic acids per gram. Ferulic, isoferulic, and caffeic acids are the major phenolic acids present in soybeans (Seo and Morr, 1984) and they have been shown to inhibit carcinogenesis in animal models (Tanaka et al., 1993) The anti- cancer activity of phenolic acids is most likely due to their antioxidant properties and to the ability of phenolic acids to reduce hyperplasia (Stich, 1991; Tanaka et al., 1993). There is insufficient data to conclude if phenolic acids reduce CC or if dietary soy can provide sufficient quantities of phenolic acids to inhibit colon carcinogenesis.

h). Phytosterols

Soybean phytosterols are found primarily in the oil fraction of the bean. Soybean oil contains about 372 mg of total phytosterols per 100 g of oil (Weihrauch and Gardner, 1978). The main phytosterols in soybeans are β-sitosterol (56%), campesterol (21%), and stigmasterol (20%). Refining the oil reduces the phytosterol content by about 30%, whereas refining followed by hydrogenation reduces phytosterol content by about 60%.

There are both epidemiologic and experimental CC studies suggesting that dietary phytosterols may help prevent CC. Epidemiologic studies indicate that there is an inverse relationship between dietary phytosterol intake and risk of CC (Nair et al., 1984; Rao and Janezic, 1992). Raicht et al. (1980) induced CC in rats with N-methyl-N-nitrosourea and showed that 0.2% dietary β-sitosterol reduced both CC incidence and the number of

tumors per rat. Awad et al. (1997) reported that β-sitosterol possibly inhibits the growth of HT-29 colon cancer cells through altered sphingomyelin metabolism. Growth inhibition was associated with increased cellular concentrations of ceramide.

Vegetarians of the Seventh-day Adventist religion (a population with low risk for colon cancer) consume about 350 mg of phytosterols per day. Soybeans contribute no more than 10% of the total phytosterol intake for Seventh Adventist vegetarians. Therefore, it is unlikely that soy phytosterols could have a major role in reduction of colon cancer risk in either vegetarians or non-vegetarians.

i). Saponins

Soybeans are the major dietary source of saponins since saponin (triterpenes) distribution is limited in commonly consumed foods. The saponin contents of whole soybean, soybean flour, tofu, soy isolate, and lecithin are 5.6, 2.2, 2.1, 0.8–2, and 2.9% of the dry weight, respectively (Oakenfull, 1981). Part of the saponins is removed during oil extraction and an additional portion is removed from the oil during refining.

Saponins are amphiphillic compounds composed of water soluble sugar residue attached to a lipid- soluble aglycone (sapogenol). The biological activity of each saponin depends upon its polarity, acidity and hydrophobicity, characteristics determined by chemical structure (Milgate and Roberts, 1995). Under invitro conditions, various saponins have demonstrated antimutagenic, anticarcinogenic and antimetastatic effects against multiple cell lines (Rao and Gurfinkel, 2000). Soyasponins encompasses three groups of sapogenols, are comprised of a neutral, non polar oleane-12-ene triterpene aglycone with differing sugar moieties linked at one or more glycosylation sites (Yoshiki

et al., 1998). Crude soybean saponin mixtures at concentrations ranging from 150 to 2400mg/L inhibit growth of multiple human colon adenocarcinoma cell lines *in vitro*. Treatment of human colon cancer cell lines with soyasaponins suppressed growth, induced morphological alterations, increased multiple markers of differentiation, and inhibited protein kinase C (PKC) activity (Sung et al., 1995a; Sung et al., 1995b; Oh and Sung, 2001). Thus, soy saponins exhibit inhibitory effects toward neoplastic cells, actions that are similar to those of various plant saponins reported to be anti-neoplastic and anti carcinogenic agents (Rao and Gurfinkel, 2000). Following consumption of soy, soy saponins appear to pass undigested through the small intestine. Saponins may be partially metabolized to sapogenols by bacterial glycosidases in the colon and the resulting mixture of saponins and sapogenols can presumably interact with the epithelium (Gestetner et al., 1968; Gurfinkel and Rao, 2003; Hu et al., 2004). Soyasaponins could therefore potentially modulate colon carcinogenesis.

A study by Koratkar and Rao, (1997) found that a diet containing 3% soy saponins, when administered 1 week after a series of AOM injections, reduced the average number of ACF per colon in CF1 mice as compared with mice consuming a control AIN93G diet (2.6 vs 7.67 ACF per colon respectively). As saponins are not absorbed and remain in the gastrointestinal tract they may facilitate binding of bile acids to fiber (Sidhu and Oakenfull, 1986). There is evidence that bile acids may act as tumor promoters in the colon (Reddy and Wynder, 1977). Therefore, reducing the concentration of soluble bile acids in the colon could potentially reduce colon cancer. Better analytical methods to quantify saponins are needed to aid in anti-cancer research of soy saponins.

2.3. Role of calcium in colorectal chemoprevention

i. Epidemiology

In the last decade there has been considerable interest in the study of calcium as a chemopreventive agent against colorectal cancer. Several epidemiologic studies show (Garland et al., 1985; Sorenson et al., 1988) that CC incidence is lower in regions of western industrialized societies where consumption of calcium-containing dietary constituents such as dairy products were relatively high. However, epidemiological data regarding the association between calcium ingestion and colorectal cancer or adenoma have varied. A number of case-control and cohort studies have suggested that increased intake of calcium and of vitamin D may be associated with a reduced CC incidence. However, other studies (Bostick et al., 1993b; Neugut et al., 1996) have not clearly supported this association. The Iowa Women's Health Study (Bostick et al., 1993b) investigated whether a high intake of calcium, Vitamin D or dairy products protected against colon cancer among women (Lointier et al., 1992; Brenner et al., 1994) over 55 years of age. The relative risks (RR) for CC observed for the highest quintile of intake as compared with the lowest were 0.52 (95% CI 0.35-0.84) for calcium and 0.54 (95% CI 0.35-0.84) for vitamin D. However, when incorporated into a multivariate model, the trends lost statistical significance and RR were attenuated (Bostick et al., 1993b). Tseng et al. (1996) evaluated the association between a number of micronutrients including calcium and the risk of colorectal neoplasia. In this study, a reduced risk of adenomas was confined to men with the highest calcium quartile. Another case-control study evaluated the association between regular usage of supplements containing vitamins A, C, D and E or calcium with the risk of developing colorectal adenomas (new or

recurrent). No protective effect was documented for any of the nutrients examined, including calcium (Neugut et al., 1996). Overall the epidemiological data on calcium intake is quite heterogeneous, suggesting a very modest beneficial effect at best.

Newmark et al. (1984) proposed a hypothesis based on the consideration that consumption of calcium leads to increase in ionic concentration of calcium in the intestinal lumen. He suggested that luminal calcium reacts with bile acids and dietary long-chain fatty acids to form insoluble complexes, thereby nullifying the cytotoxic effects of these lipids. Several experimental animal studies conducted by Wargovich et al. (1983; 1984) have shown that calcium can ameliorate the damaging effects of these agents when applied intra-colonically. These findings were supported in later animal studies, which found that calcium prevented the cytotoxic effect of intestinal lipids and prevented increased in intestinal cell proliferation, thereby influencing the development of colorectal cancer (Bird, 1986; Rafter et al., 1986).

ii. Mechanism

A number of mechanisms have been postulated to explain the putative chemopreventive effect of calcium against CC development. Calcium may act through the binding of bile and fatty acids in the intestinal lumen and decreasing exposure of the intestinal epithelium to those substances. Bile and fatty acids can produce toxic effects to the colonic epithelium and cause increased cell proliferation or compensatory hyper-proliferation secondary to repair responses (Bostick et al., 1993a). In either case, increased proliferation may ultimately lead to carcinogenesis of the colonic epithelium. Typically, 40 to 90% of ingested calcium is not absorbed in the small intestine and can

form calcium salts with bile acids or fatty acids. Calcium salts of bile acids may be less irritating to the colon mucosa than bile salts formed with sodium or potassium (Wargovich et al., 1983). Therefore, when high fat diets are consumed and there are more bile acids passing through the colon, high levels of dietary calcium provide more calcium potentially available to complex with bile acids. Since calcium salts of bile acids are less irritating to the colonic mucosa, epithelial cell proliferation is reduced. Thus, calcium could act in a nonspecific, non-cellular manner to reduce colon cancer incidence by reducing cell proliferation.

Calcium may also directly influence colonic epithelial cell proliferation (Lipkin and Newmark, 1985) or induce terminal differentiation of colonic epithelial cells (Newmark et al., 1984). Increasing the intraluminal concentrations of calcium in the colon may produce results (decreased cell proliferation and increased cell differentiation) similar to those observed when colon tissue explants and cells were cultured in medium containing 2.2 mMol/L calcium (Buset et al., 1986). These data suggest that increased intraluminal calcium concentrations may decrease cell proliferation and increase cell differentiation by acting directly on mucosal cells (Buset et al., 1986). Furthermore, it has been reported that addition of the calcium ionophore A23187 to cell cultures stimulates proteolysis of the APC protein and induces apoptosis (Browne et al., 1994). High levels of dietary calcium can attenuate the increased cell proliferation and tyrosine kinase activity caused by azoxymethane injections in rats (Arlow et al., 1989). These observations suggest that intraluminal calcium can directly affect mucosal cells in a manner that is independent of bile salts.

Data from the large prospective studies on calcium and colon cancer risk show weak, non-significant associations, although there is some evidence of a dose-response relationship (Martinez and Willett, 1998). Some clinical studies have demonstrated an anti-carcinogenic effect as a result of calcium supplementation. Calcium intervention trials in humans (Lipkin et al., 1989; Rozen et al., 1989; Steinbach et al., 1994) have shown that supplementing human diets with calcium can reduce intestinal cell proliferation (an intermediate biomarker of colorectal cancer risk) in the upper 40% of colon crypts in people at intermediate or high risk of colon cancer. However, other studies (Gregoire et al., 1989; Stern et al., 1990) have shown no effect of dietary calcium, whilst one (Kleibeuker et al., 1993) showed the opposite effect. A review of 17 human studies of calcium supplementation and colorectal epithelial cell proliferation concluded that it is unlikely that calcium supplementation can substantially lower total labeling indices, but it may normalize the distribution of proliferating cells within colon crypts (Bostick, 1997). A randomized placebo-controlled study reported no effect of calcium supplementation on rectal mucosal proliferation in patients with previous colon adenomas (Baron et al., 1995). A double-blind three-year intervention study with calcium (1.6 g/day) and other anti-oxidants in polyp-bearing patients (n = 116) also reported that there was no overall effect of calcium on polyp growth. However, the study reported that calcium and the antioxidants prevented formation of new adenomas (Hofstad et al., 1998).

The effects of calcium supplementation on bile acid content also have been evaluated clinically. Two different studies documented beneficial calcium-induced changes in the bile acid composition, which were characterized by a decrease in total as

well as secondary bile acid levels in feces (Alberts et al., 1996; Lupton et al., 1996). A randomized placebo-controlled phase III study (Baron et al., 1999) demonstrated a moderate (20%) but significant reduction in the incidence of new adenomas seen in the cohort receiving calcium supplementation (1200 mg/d elemental calcium). The adenoma recurrence rate in the calcium treatment group was 31% and that of the placebo group was 38% (Baron et al., 1999). Similar results, though not statistically significant, were observed in the smaller European Calcium Fiber Polyp Prevention trial (Faivre et al., 1999). A recently published analysis of cases from the Nurses' Health Study and the Health Professionals Follow-Up Study found that calcium reduced the risk of distal colon cancer but not of proximal colon cancer (Wu et al., 2002). The study also suggested a threshold effect whereby most of the benefit was achieved by reaching intakes of 700 to 800 mg/day. Overall, the potential benefical effects of calcium in reducing colon cancer risk appear modest.

In rodent models of chemically-induced colon carcinogenesis (promoted by high fat diets), calcium administration was associated with protection against colorectal carcinogenesis as evidenced by decreased epithelial cell proliferation, ACF numbers and tumor formation. High calcium diets (10 g/kg) normalized the distribution of proliferating colon cells in rats treated with N-methyl-N'-nitro-N-nitrosoguanidine (Reshef et al., 1990) and inhibited azoxymethane-induced cell proliferation (Li et al., 1998). Other studies have demonstrated that high-calcium diets reduced aberrant crypt formation (Pereira et al., 1994; Li et al., 1998; Pierre et al., 2004) and chemically-induced colon cancer in rats (Appleton et al., 1987; Beatty et al., 1993; Quilliot et al., 1999). Recent studies in rodent models have further demonstrated that calcium decreases colonic tumors

induced by targeted mutations (Yang et al., 1998) or by dietary factors (Newmark et al., 2001). Thus, several studies in rodent models have demonstrated that dietary calcium can reduce development of CC.

E. Biomarkers and models for chemopreventive studies

1. Surrogate end point biomarkers (SEB)

Biomarkers of carcinogenesis are quantifiable molecules or changes involved in physiological or pathological events which occur between exposure to exogenous or endogenous carcinogens and the subsequent development of cancer (Kelloff et al., 1994). They can be categorized as molecular, histological, risk and tumor biomarkers. Some of the biomarkers of early colorectal carcinogenesis can provide a means for diagnosing very early changes associated with cancer development before actual tumors or even polyps are present.

These biomarkers are increasingly used in programs of screening, chemoprevention and chemotherapy for CC. Such biomarkers are termed 'surrogate endpoint biomarkers' (SEBs) and have been identified for several different stages in the carcinogenic process. These biomarkers represent a means of monitoring disease progression without having to wait for true neoplasia and metaplasia to develop. SEBs are also termed 'preneoplastic biomarkers' and should be distinguished from 'risk biomarkers' and 'tumor markers'. Of these three types of markers, preneoplastic biomarkers are the most useful for assessing the efficacy of cancer chemopreventive agents (Einspahr et al., 1997; Collet et al., 1999; Sharma, 2000). Examples of cellular

preneoplastic biomarker measurements include those of proliferation, differentiation and apoptosis.

2. Cell proliferation biomarkers

Increased epithelial cell proliferation is a common feature of colon carcinogenesis. Since the discovery of cell-cycle associated proteins such as PCNA and Ki-67, their expression has been used in several studies (Renehan et al., 2002) for IHC detection of proliferating cells. Normally, proliferation in colonic crypts is restricted to the basal 60% of the crypts. As colonocytes migrate up the crypt, these cells undergo terminal differentiation. Ultimately, after approximately 7 days of transit time to the crypt surface, colonocytes undergo programmed cell death and are extruded. Under normal circumstances, cell proliferation does not occur in the upper 40% of the crypts (Lipkin, 1988). An expansion of the proliferative compartment toward the crypt surface (hyperproliferation in the middle and upper thirds of the crypt) is considered a preneoplastic biomarker (Lipkin, 1988) and has been described in association with development of adenomatous polyps and carcinoma (Risio et al., 1991).

Methods used to assess colonic epithelial cell proliferation can be broadly divided into 'static techniques' that reflect the proliferation state (percentage of a proliferation-associated population at any particular time within the colonic crypts) and 'dynamic techniques' which reflect cell proliferation rate (including cell cycle phase transit time) (Renehan et al., 2002). 'Dynamic' measurements are more biologically relevant. However, their assessments require either systemic administration of radioactive agents, or *in vitro* incubation of tissue samples with tritiated thymidine or bromodeoxyuridine

(BrdU). Therefore, static measurements such as S-phase fraction analysis using flow cytometry and immunohistochemical (IHC) measurements of cell cycle-associated intrinsic proteins such as PCNA and Ki-67 antigen are preferred methods because of their ease of use in studies having large numbers of specimens.

PCNA is an evolutionarily-conserved nuclear protein (36 kDa) identified as an ancillary factor for DNA delta polymerase. It is necessary for DNA replication and repair during cell cycle progression. PCNA is detected in cells even at G_0 and progressively accumulates during the G_1 to M phases of the cell cycle (Prelich et al., 1987; Toschi and Bravo, 1988; Zacchetti et al., 2003). Ki-67 is a nuclear antigen that is primarily expressed during S-phase of the cell cycle (Bruno and Darzynkiewicz, 1992). Therefore, Ki-67 labeling indices reflect the number of actively proliferating cells within the crypt, whereas PCNA indices are indicative of the capacity of cells to continue proliferation.

PCNA and Ki-67 proliferation indices have been used to assess the efficacy of chemopreventive agents in CC studies and in clinical studies that evaluate the risk for CC in humans (Baron et al., 1995; Weaver et al., 2000; Grubben et al., 2001; Pfeiffer et al., 2003; Krishnan et al., 2004). In particular, PCNA expression (as measured by immunohistochemistry) has been used in human studies to demonstrate a step-wise increase in proliferation indices during neoplastic progression of colonic lesions (Shpitz et al., 1997), for predicting the potential for metastases in invasive colorectal carcinoma (Tanaka et al., 1995; Choi et al., 1997), and to predict the development of metachronous adenomas (Ottaviani et al., 1999). Studies also have demonstrated increased PCNA labeling indices by activation of growth factors such as EGF receptor kinase and TGF-α (Malecka-Panas et al., 1997; Barnes et al., 1998) and by overexpression of cyclins such

as Cyclin D (Izawa et al., 2002) in colonic tissue. Furthermore, several studies have demonstrated that PCNA is an accurate marker for the proliferative activity in colonic crypts, and also correlates well with other proliferation markers (e.g. BrdU) in both normal and neoplastic colonic epithelium in humans (Kubota and Kino, 1995; Tanaka et al., 1995; Yoshikawa and Utsunomiya, 1996; Choi et al., 1997; Shpitz et al., 1997; McShane et al., 1998; Yerly-Motta et al., 1999; Wu et al., 2000; Díez et al., 2003).

3. Differentiation markers

Cellular differentiation is evaluated through the expression (or lack thereof) of markers that reflect epithelial maturation. Propensity towards an undifferentiated phenotype is characteristic of neoplastic cells. Molecules that are expressed in a fully-differentiated epithelial cell may be used as a marker of the pro-differentiating effects of an intervention. For example, *Dilidros biflorus agglutinin* (DBA), which labels mucin in goblet cells, has been used as a marker for colonic epithelial cell differentiation. Undifferentiated cell phenotypes are associated with failure of oligosaccharide synthesis and a decrease in DBA staining (Boland et al., 1988). Reduced DBA staining has been documented in colon adenomas and cancers as well as some familial colon cancer syndromes (Sam et al., 1990).

4. Apototic markers

Homeostasis of the normal colonic epithelium requires a proper balance between cell proliferation and cell death (Hall et al., 1994; Ahnen, 1999). When colonocytes migrate to the luminal surface, they undergo a process of physiologic cell death termed

apoptosis and are sloughed into the intestinal lumen. Apoptosis is associated with marked alterations of cell surface structure (such as reduced cell adhesion) so that cells lose contact with their neighbors and be sloughed early in the process of apoptosis. Failure of apoptotic function is a characteristic of neoplastic phenotypes and may play a role in tumor progression (Bedi et al., 1995).

No markers of cell sloughing exist, but several markers of apoptosis have been applied to the colon mucosa. However, quantitation of apoptotic end points in colonic epithelium has proven difficult. This is primarily due to the relatively low rates of apoptosis observed in colonic epithelial cells and the rapid rate of cell loss after initiation of apoptosis. Many investigators have used detection of fragmented DNA, a characteristic of apoptotic cells, and measured by TUNEL immunohistochemistry. Other methods for detecting apoptosis in human colonic crypts (flow cytometry, caspase induction, Bcl-2/Bax ratio) have not been sufficiently explored to be useful as reproducible biomarkers of apoptosis in the human colonic crypt. Apoptosis as a biomarkers for chemopreventive activity in morphologically normal human colonic epithelium, while of interest and potential importance, remains unvalidated.

5. Aberrant crypt foci

The smallest recognizable histopathologic evidence of colorectal epithelial carcinogenesis is the ACF or microadenoma. ACF are lesions consisting of large thick crypts as observed in methelene blue-stained specimens of colon and were originally found in mice treated with AOM (Bird, 1987). In humans, two types of ACF are observed: hyperplastic foci (hypercellular but normal appearing cells) and dysplastic foci

(contain dysplastic cells similar to those found in adenomas) (Polyak et al., 1996; Siu et al., 1997; Takayama et al., 1998). Hyperplastic lesions commonly contain K-ras mutations but rarely have APC mutations (Pretlow et al., 1993; Jen et al., 1994; Smith et al., 1994) and are unlikely to progess to neoplasia. Dyplastic lesions, on the other hand, are thought to be precursors to adenomas. Numerous dysplastic ACFs are found in FAP patients (Hamilton, 1983). Moreover, the occurrence patterns of ACF and their response to chemopreventive interventions parallel those of adenomas and carcinomas in rodent carcinogenesis models (Bird, 1987; Wargovich et al., 1995b; Reddy et al., 1996; Kawamori et al., 1998). Thus, ACFs represent an excellent early surrogate marker of CC risk to assess in rodent models and potentially, in humans.

6. Animal models

Animal models represent a pivotal tool in the efficacy evaluation of chemopreventive agents. Chemical carcinogenesis in rodent models provides reproducible development of tumors in the intestinal epithelium of rodents that have been exposed to a chemical compound that can act as a complete carcinogen. Frequently-used chemical carcinogens include dimethyl hydrazine (DMH), azoxymethane (AOM), and methoxymethane. DMH undergoes conversion into AOM and azoxymethanol, which are subsequently conjugated with glucuronic acid and excreted in bile. Bacterial hydrolysis occurs once the conjugated carcinogens reach the intestinal lumen but is not essential to their tumorigenic effect (Hartiala, 1973; Fang and Strobel, 1978).

The azoxymethane-injected rat model is considered to be a highly relevant colon tumorigenesis model system. Although the specific etiology of CC and other diet-

associated cancers remain unclear, the chemical carcinogen-induced rat colon tumor system is a valuable model for studying the potential mediators of colonic cell proliferation, differentiation and apoptosis, which are useful preneoplastic biomarkers of colon cancer. Evidence to support use of this model includes: i) utilization of AOM provides a clear distinction between tumor initiation and promotion; ii) carcinogen-induced K-ras and APC mutations occur as early events in colon carcinogenesis, similar to humans; iii) the development of tumors is responsive to the amount and type of dietary fat, and iv) AOM-induced colon tumors closely parallel human colonic neoplasia in pathologic features (Ahnen, 1985; Reddy et al., 1991; Kakiuchi et al., 1995; Chapkin et al., 1997; Maltzman et al., 1997). In addition, the similarity between the expression of intracellular modulators of apoptosis (β-catenin, COX-II, bcl-2, bax) in AOM injected rats and in humans suggest that this experimental system is a good model for studying mechanisms that regulate apoptosis in humans (Dubois et al., 1996; Hirose et al., 1997; Takahashi et al., 1998). AOM also induces changes (such as formation of ACF, adenomas and adenocarcinomas) in the rodent colon that closely replicate, histologically, the adenoma-carcinoima sequence in humans (Ward et al., 1973). Thus, AOM-induced rat colonic neoplasms are similar to human colonic tumors in their histologic features and proliferation characteristics. Given the similarities in gene mutations and gene expression that exist for rodent and human colon carcinogenesis, this model is a highly relevant model for preclinical *in vivo* testing of new chemopreventive agents.

F. Conclusion

Colon cancer is a leading contributor to cancer prevalence and mortality in the Western world (Parkin, 2004), accounting for 15% of all cancers diagnosed annually in the U.S. (American Cancer Society, 2005). Identification and application of effective prevention and treatment strategies for this disease represent crucial public health challenges in industrialized countries. Diet has been recognized as an important modifiable risk factor for CC (AICR/WCRF, 1997). Diet may contain chemopreventive agents such as soy and calcium. These agents can inhibit the initiation of preneoplastic lesions by carcinogens or reverse their progression to invasive cancers. Dietary patterns that emphasize legumes as protein alternatives to meat also may reduce CC risk (Byers et al., 2002).

Soybeans have been extensively studied and found to be protective against CC in various model systems. These results are supported by epidemiological studies on Asian populations and immigrants which demonstrate a protective role of soy consumption against CC risk. While the protective role of soy has been attributed in part to its high phytochemical content (e.g. genistein, daidzein, BBI), studies on the efficacy of isolated soy phytochemicals to protect against CC have yielded mixed results. It is unlikely that the efficacy of soybeans against CC risk can be attributed to one chemical constituent, but rather to the synergistic effects of a composite of several chemopreventive components found in soy.

Despite many epidemiogical studies on the potential of calcium to prevent colorectal cancer, a protective effect has been difficult to establish. Recent prospective studies and randomized controlled studies on calcium intake and CC risk support a

modest protective effect of about 20-30%. Since the inverse relationship between calcium intake and CC risk depends upon variables such as colon subsite, amount of calcium and vitamin D exposure, future studies will be needed to examine these variables. There is significant biologic plausibility for a relationship between increased calcium intake and reduced CC risk and, because calcium is associated with benefits to other organ systems, calcium supplements should be considered in chemoprevention strategies for CC.

The use of colorectal cancer incidence as an end point in chemoprevention trials is impractical due to the long time frame of colon carcinogenesis as well as the large numbers of subjects required to test the efficacy of various interventions. Thus, there is a need to identify easily-detectable biomarkers of a precancerous state as intermediate biomarkers that can reliably predict CC risk and possible benefits of intervention in chemoprevention trials (Einspahr et al., 1997).

Once identified, potential preneoplastic biomarkers must be painstakingly validated. This task involves the judicious use of the most suitable animal models which reflect the multifactorial nature of colorectal cancer (Shureiqi et al., 2000). Furthermore, the relationship between the biomarker and risk of cancer has to be established in prospective studies in high-risk individuals. Such studies constitute a significant undertaking, but are necessary to bridge the gap between preclinical model systems and clinical trials.

Among the methods proposed as surrogate end-point biomarkers for the assessment of CC risk in human populations, the assessment of colon epithelial cell proliferation and ACF visualization *in vivo* are very promising. Cell proliferation can be repeatedly evaluated by obtaining biopsy material routinely taken during sigmoidoscopy.

These techniques are both relatively easy and of low cost. ACF visualization *in vivo* seems quite promising as an intermediate biomarker of CC risk, but this technique requires further validation. At the present level of knowledge it appears that these approaches could be used in conjunction for evaluating CC risk in human populations and in intervention trials.

CHAPTER III. CELL PROLIFERATION INDICES IN COLONIC CRYPTS OF HUMANS AT VARYING AGES AND RISKS FOR COLON CANCER

A. ABSTRACT

Colon cancer (CC) intervention studies require a sensitive and accurate index to measure treatment effectiveness. Mucosal cell proliferation before and after treatment is frequently used to monitor effectiveness. This study was conducted to determine the correlations between CC risk (subjects varying in age and history of polyps, familial adenomatous polyps-FAP or CC) and 3 indices of mucosal proliferation: labeling index (LI), proliferation zone (PZ) and relative proliferating cell nuclear antigen (PCNA) levels. Subjects (46) were grouped based on age, 20-29 yrs (n=6, mean age of 22.7 ± 3.1 years), 30-49 yrs (n=6, mean age of 38.9 ± 6.7 years), 50-69 yrs (n=6, mean age of 59.3 ± 5.9 years), 70-79 yrs (n=7, mean age of 72.1 ± 1.9 years) and clinical history, adenomas (n=8, age range of 44 to 72 years with mean age of 57.9 ± 12.4 years), FAP (n=7, age range of 9 to 46 years with mean age of 23.6 ± 13.5 years, 3 males, 4 females) and CC (n=6, age range of 52 to 74 years with mean age of 65.7 ± 9.0 years). Cell proliferation in the colonic biopsies was measured by PCNA and Ki-67 immunohistochemistry and relative PCNA levels by slot-blot quantification. The results showed that the PCNA LI, PCNA PZ, Ki-67 PZ and relative PCNA levels increased with age and history of polyps, FAP or CC (at $P<0.05$). There were no significant differences in the Ki-67 total LI among subjects at varying age and history of colonic diseases. This study shows that the proliferative compartment (as assessed by PCNA and Ki-67) and relative PCNA levels, but not Ki-67 LI, were positively correlated with increasing age and CC risk, and

therefore useful discriminators to assess CC risk in large scale clinical studies and chemo-modulation in dietary intervention studies.

B. INTRODUCTION

One of the early events in the multi-stage process of colon carcinogenesis is an increase in colonic cell proliferation (Vogelstein et al., 1988; Vogelstein and Kinzler, 1993). This is supported by observations that hyper-proliferation occurs in colonic crypts of subjects at elevated risk for colon cancer (CC) (older age, presence of polyps, family history of CC, ulcerative colitis, Familial Adenomatous Polyposis) when compared to young subjects with no history of colon disease (Lipkin, 1984; Lipkin, 1987; Deschner et al., 1988; Roncucci et al., 1988; Paganelli et al., 1990; Rozen et al., 1990; Paganelli et al., 1991; Risio et al., 1991; Gerdes et al., 1993; Bostick et al., 1995; Bostick et al., 1997b; Mathers et al., 1998; Mills et al., 2001; Sinicrope et al., 2004). Indices of cell proliferation (Labeling Index [LI]-fraction of labeled cells in colonic crypt and Proliferation zone [PZ]-highest labeled cell in colonic crypt) in the colonic mucosa and the distribution of the proliferating cells in the mucosal crypts are commonly used as surrogate biomarkers of CC risk in clinical trials. Methods typically used in the past to assess colonic cellular proliferation have included *in vivo* administration of tritiated thymidine and BrdU, (to label cells in the S-phase) ex-vivo incubations of colonic biopsies with BrdU and, more recently, the use of endogenous markers such as proliferating cell nuclear antigen (PCNA) and Ki-67 antigen. PCNA is a nuclear protein identified as an ancillary factor for DNA delta polymerase and detected in all phases of

the cell cycle (Bravo and MacDonald-Bravo, 1987). Ki-67 is primarily expressed during S-phase of the cell cycle and is absent in the G_0 phase (Bruno and Darzynkiewicz, 1992).

In normal colonic mucosa, proliferating cells are restricted to the basal 60% of the crypts (Lipkin, 1988). Previous research by Lipkin (1971; 1975; 1987) and Deschner (1975; 1975; 1977; 1985) consistently found increased proliferation indices (both LI and expansion in PZ) in normal mucosa of subjects with risk factors for CC (family history of polyposis, previous history of CC and sporadic adenomas) when compared to normal controls of similar age ranges. Studies using either BrdU or tritiated thymidine to label proliferating cells have demonstrated hyper-proliferation and expanded proliferative zones in macroscopically normal rectal mucosa of persons with adenomas (Wilson et al., 1990; Risio et al., 1991), expanded proliferative zone in persons with a strong family history of CC (Gerdes et al., 1993), and an increase in LI (mean mitosis/crypt) in macroscopically normal colonic mucosa of persons with sporadic adenomas, carcinomas or FAP when compared to normal subjects (Mills et al., 2001). Deschner et al. (1988) observed that increased CC risk is more closely related to an expansion in the PZ rather than to total crypt LI. Subsequently, several other studies also have indicated that expansion of the PZ, rather than total crypt epithelial cell proliferation, is a better indicator of increased CC risk (Paganelli et al., 1991; Risio et al., 1991; Gerdes et al., 1993; Mills et al., 1995; Paspatis et al., 1998; Akedo et al., 2001).

Some researchers have demonstrated inconsistent observations with colonic epithelial proliferation indices and thus question the relevance of LI and PZ as indicators of the CC risk (Biasco et al., 1994; McShane et al., 1998). Keku et al. (1998) found inconsistent association between CC risk, diet and rectal proliferation indices in subjects

with adenomas. Sandler et al. (2000) reported that proliferative index did not predict future colorectal neoplasia and was weakly associated with current history of adenomas in humans. Differences among the various studies that have examined colonic epithelial cell proliferation have included variable methods for tissue collection and preparation (e.g. culture of biopsies for tritiated thymidine and BrdU labeling) and, more importantly, the use of different endogenous or exogenous proliferation markers (e.g. PCNA, Ki-67, tritiated thymidine, BrdU, and S-phase fraction by flow cytometry). Each of these markers detect cells in S-phase and some of these markers (e.g. PCNA, Ki-67) label cells in other phases of cell cycle. Therefore, the various markers measure different aspects of cell proliferation cycle and lead to inherently variable observations (Renehan et al., 2002).

Aging is characterized by an overall increase of epithelial cell proliferation in colorectal mucosa and by an upward expansion of the proliferative compartment, similar to that observed in populations at increased risk for colon cancer. Deschner et al. (1988) reported that LI (using tritiated thymidine) in normal mucosa of rectal biopsies of older persons with no colonic disease (mean age of 55.9 ±14.3, LI = 7.7%) was significantly greater than that observed in younger persons who had no history of colonic disease (mean age of 23.3 ± 1.5, LI = 5.8%). Roncucci et al. (1988) used tritiated thymidine labeling to demonstrate an overall increase in rectal epithelial cell proliferation and an expansion of the proliferative compartment with increasing age of normal mucosa of subjects who were grouped into categories of 30 to 51, 51 to 65 and 66 to 90 years of age. Sjöqvist et al. (2002), using DNA-flow cytometry, compared S-phase fraction of colonic

epithelial cells of middle-aged persons (mean of 41± 8.9 years) and an older age group (mean of 69 ± 8.4 years) and reported that the S-phase fraction increased with age.

To date, there has been no published research which compares PCNA and Ki-67 proliferation indices in persons of widely varying ages who have no history of colonic diseases. The first objective of this study was to use immunohistochemical methods to assess and directly compare PCNA and Ki-67 proliferation indices in colonic mucosa biopsies obtained from persons who differed in age and known risk factors for CC development. Given the laborious nature of IHC techniques used to measure proliferation markers, there is a need to develop more facile indices of colonic epithelial cell proliferation. Simpler methods would be highly desirable in dietary intervention and large scale clinical studies that evaluate the risk for CC. For this reason, the second objective of this study was to use an immunoblotting method to determine relative levels of PCNA protein in colonic epithelial cells and determine if these correlate with proliferation indices based on IHC techniques.

C. MATERIALS AND METHODS

Experimental design

A total of forty-six subjects (20 males and 26 females) were recruited by the Division of Gastroenterology at Michigan State University (MSU) clinical center. All subjects received a remuneration of $ 50 for participation in the study and the study was approved by the University Committee on Research Involving Human Subjects at MSU.

Subjects were divided into two broad classes a) subjects without a history of colonic diseases (adenomas, FAP or colon cancer) were considered 'controls' and b)

subjects with a history of colonic diseases were considered at risk for CC. Control subjects were referred for endoscopic examination for health maintenance or volunteers from MSU. They were divided into four age ranges, 20-29 (n=6, mean age of 22.7 ± 3.1 years, 2 males, 4 females), 30-49 (n=6, mean age of 38.9 ± 6.7 years, 3 males, 3 females), 50-69 (n=6, mean age of 59.3 ± 5.9 years, 4 males, 2 females) and 70-79 (n=7, mean age of 72.1 ± 1.9 years, 3 males, 4 females) with an overall mean age of 49.2 ± 20.0 years. Subjects (overall mean age of 48.7± 11.6 years) with colonic diseases were divided into three additional groups on the basis of their history of neoplasia. The groups were, persons with a history of adenomatous polyps (n=8, age range of 44 to 72 years with mean age of 57.9 ± 12.4 years, 1 male, 7 females), persons with a history of FAP (n=7, age range of 9 to 46 years with mean age of 23.6 ± 13.5 years, 3 males, 4 females) and persons with a history of CC (n=6, age range of 52 to 74 years with mean age of 65.7 ± 9.0 years, 4 males, 2 females, three subjects had undergone surgical resections of their colons in the past). Biopsies were obtained from the same site (30-40 cms from the anal verge) for all subjects except for two subjects in the FAP group (who had colostomies and biopsies were obtained from the rectal stump).

Tissue procurement and processing

All subjects received an oral electrolyte bowel preparation, Golytely^{TM} containing polyethylene glycol 3350^{TM}, NaCl, KCl, $NaHCO_3$, and Na_2SO_4, the evening before flexible sigmoidoscopy or colonoscopy. Colonic biopsy samples (4) per subject were obtained during flexible sigmoidoscopy or colonoscopy. Biopsies approximately 3mm by 8mm were obtained from normal appearing mucosa 30 to 40 cms from the anal verge

using jumbo forceps. All biopsies were immediately rinsed in phosphate buffered saline (pH 7.4). Two samples were placed individually in 0.6 ml microcentrifuge tubes and immediately frozen on dry ice for subsequent slot-blot quantification of PCNA. The remaining samples were pinned flat to cardboard strips and fixed in B5 fixative for 1 hour and in neutral buffered formalin for 2 – 6 hours. The fixed biopsies were placed between foam pads in histopath cassettes, dehydrated, infiltrated with paraffin and embedded in paraffin using standard histologic procedures.

PCNA and Ki-67 IHC analyses

Three micron thick sections of paraffin-embedded biopsy samples were cut and mounted on poly-L-lysine coated glass slides and dried at 58° C for 2 hours. Sections were cleared of paraffin with xylene, hydrated in increasing ratios of water:ethanol, and treated with iodine:potassium iodide and 5% sodium thiosulfate to remove mercury. Samples were then subjected to antigen retrieval (10 mMol/L citrate buffer, pH 6.0, 95 °C) for 20 minutes before immersion in 0.3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity. Following rinses in tris-buffered saline (TBS), sections were then incubated with primary antibody (1:100 dilution of PC10 [anti-PCNA] or MM1 [anti- Ki-67] respectively in 1% BSA in Tris buffered saline, pH 7.4) overnight at 4 °C. After subsequent incubations in biotinylated rabbit anti-mouse immunoglobulins (diluted 1:100, 45 min), followed by peroxidase-conjugated streptavidin (diluted 1:300, 45 min), they were treated with 3-amino-9-ethylcarbazole (AEC) and color development was monitored for 10–20 minutes until red staining was evident. The sections were rinsed twice with 10 mMol/L TBS in between incubations with antibodies, streptavidin

and AEC. Slides were then counter-stained with hemotoxylin and cover-slipped using Faramount mounting medium. Appropriate positive (human tonsil) and negative controls (omission of primary antibody) were used for evaluation of PCNA and Ki-67 staining. The primary antibodies were obtained from Novacastra (now Vector Laboratories; Burlingame, CA), and biotinylated rabbit anti-mouse immunoglobulin, streptavidin, AEC, and Faramount were obtained from Dako (Carpinteria, CA).

Quantification of PCNA and Ki-67 IHC:

PCNA and Ki-67 labeling were quantified by the percentage and positions of positively stained (red) cells within full-length crypts in colonic sections. Well-oriented longitudinally-sectioned full-length crypts (n = 10 per subject) were identified by light microscopy. A scorable crypt was defined as one in which the base touched the muscularis mucosa and had an open lumen at the top. The number and position of PCNA and Ki-67 labeled cells and crypt heights were recorded for ten full crypts for each subject to determine cell proliferation parameters. All cells with a red nuclear staining were counted as positive. The center-most cell at the base of the crypt, and in between the hemicrypts, was designated as cell number one and the cell at the top of the crypt mouth was considered the highest cell. A continuous column of cells from cell number one to the highest cell were counted along each hemicrypt wall of each crypt. Crypt height was defined as the number of cells per hemicrypt.

Total Crypt Labeling Index (LI) was calculated by dividing the number of labeled cells in a hemicrypt by the total number of cells in a hemicrypt (crypt height). These values were averaged for at least 20 hemicrypts (representing 10 full-length crypts) to

obtain the LI for each subject. Compartment Labeling Indexes (basal LI, mid LI and top LI) were calculated by dividing the number of labeled cells within each third (basal, middle and top) of each hemicrypt by the number of cells in each third of the respective hemicrypt. Proliferation zone (PZ) was obtained by dividing the position of the highest labeled cell from the hemicrypt base by the total number of cells in the hemicrypt. Each of these PZ values were averaged for at least 20 hemicrypts to obtain the overall PZ for each subject.

PCNA Measurement by Slot-blot

Colonic biopsy samples were homogenized with 0.5ml of non-denaturing buffer containing protease inhibitors (20mMol/L MOPS, 150 mMol/L sodium chloride, 1% vol/vol Triton X-100, 1% wt/vol deoxycholate, 0.1% wt/vol SDS, 1 mMol/L EDTA, 100 μMol/L sodium orthovandate, 250 μMol/L PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin) and total protein concentrations were determined by the biocinchoninic assay using BSA as the standard. Relative PCNA levels in tissue homogenates were then determined by immuno-quantification using slot-blot methodology. PVDF membranes (Immobilon P membranes; Millipore, Billerica, MA) were wetted in methanol and then transferred to PBS. Twenty μg of protein from each sample (dilutions in PBS) was vacuum blotted on to the PVDF membranes using a slot-blot apparatus (Hoefer Scientific instruments). The membranes were held overnight at 4 °C in blocking buffer (3% BSA in TBS with Tween 20). The membranes were then incubated for 2 hours with monoclonal PC-10 antibody (1:1000 dilution of primary antibody in blocking buffer). After rinsing with wash buffer (TBS with Tween 20) the membranes were incubated in biotinylated secondary antibody

(1:1000 dilution of goat anti-mouse IgG in wash buffer for one hour). After subsequent rinses with wash buffer, the membranes were treated with a chemiluminescence reagent (Dupont NEN research products) and light emission was captured by autoradiography. Developed autoradiography films were quantified by scanning densitometry. Known amounts of mouse IgG Fc fragments standard curve ranging from (0.3125ng to 10.00ng) were included in each blot to allow for normalization of staining intensity across blots.

Statistical Analysis

Crypt height, PCNA and Ki-67 proliferation indices (total crypt LI, PZ, and compartmental LI for basal, middle, and top compartments of the crypts) and relative PCNA quantified by slot-blot method were analyzed as a completely randomized design (one-way Analysis of variance) according to the General Linear Models procedure of SAS (version 6.11). When significant group effects were detected ($P < 0.05$), the least significant difference was used to compare group means. Simple correlation coefficients between PCNA and Ki-67 labeling indices and the relative PCNA levels and were calculated and tested for significance at $P < 0.05$ using the correlation procedure of SAS.

D. RESULTS

Proliferation indices with IHC:

Total Crypt Labeling Index (LI)

Total crypt PCNA LI (Table 3) was significantly influenced by age and risk for CC. Among subjects with no history of colonic disease, total crypt PCNA LI in the two oldest age groups (50-69 years and 70-79 years, 29% and 30% of the total cells in the

crypt had PCNA label, respectively) was significantly greater than that observed in subjects in the younger age groups (20-29 years and 30-49 years, 20% and 23% of the total cells in the crypt had PCNA label, respectively). Among control subjects with no history of colonic disease, there was a linear increase in PCNA total LI with increasing age (individual values, $r = 0.81$, $P<0.0001$, data not shown).

The total crypt PCNA LI of subjects in any of the at-risk categories were significantly greater than that observed for all control subjects regardless of age. The total crypt PCNA LI in subjects with FAP (40% of the total cells in the crypt had PCNA label) was significantly greater than that observed in any other groups.

Total crypt Ki-67 LI (Table 3) was not influenced by age or risk category and averaged 0.080 across all groups.

Proliferation zone

PCNA proliferation zone (Table 3) was significantly influenced by age and risk for CC. Among control subjects with no history of disease, PCNA proliferation zone was significantly greater in older subjects (50-69 years [PZ=49%] and 70-79 years [PZ=56%]) when compared to subjects in the younger age groups (20-29 years [PZ=37%] and 30-49 years [PZ=40%]). There was a linear increase in PCNA proliferation zone with increasing age ($r = 0.86$, $P<0.0001$, data not shown).

PCNA proliferation zone was statistically similar for older subjects (50-69 and 70-79 years) with no history of colon disease and subjects having a history of adenomatous polyps (PZ=55%) or colon cancer (PZ=55%). Among all age and risk

categories, the largest PCNA proliferation zone was observed in subjects with FAP (PZ=63%).

Ki-67 proliferation zone (Table 3) was significantly influenced by age and colonic disease. Control subjects in the oldest age group (70-79 years) had a greater Ki-67 proliferation zone (PZ=41%) when compared to control subjects aged 20-29 and 30-49 years (PZ = 28% and 31%, respectively). Among control subjects, there was a linear increase in Ki-67 proliferation zone with increasing age (individual values, $r = 0.63$, $P<0.0001$, data not shown).

Control subjects in the two oldest age groups and subjects in all disease groups had statistically similar Ki-67 proliferation zones (PZ=35%-41%) and proliferation zones in each of these groups were significantly greater than that observed in the youngest (20-29) normal subjects (PZ=28%).

Compartmental Labeling Indices (basal LI, mid LI, top LI)

Given the observed changes in total crypt PCNA LI and PZ, we were interested in determining if PCNA labeling patterns in specific regions of the crypts were influenced by age and colonic disease. PCNA labeling was observed primarily in the basal compartment, with a smaller amount detected in the middle of the crypts and very little in the top compartment (Table 4). The basal compartment LI was significantly influenced by age and risk category, but the overall pattern differed somewhat from that observed for total crypt PCNA LI (Table 3). Among the control subjects, those in the 50-69 year old age category had the greatest basal LI (58% of the cells in the basal compartment had PCNA label) when compared to subjects in the rest of the age groups. We observed a

quadratic effect of age on PCNA basal LI ($P < 0.05$). This quadratic effect was due to middle-aged subjects (50-69 years) having greater PCNA basal LI than either the youngest or oldest subject. Subjects with a history of CC (58%) and FAP (57%) had significantly greater basal LI values compared to subjects with a history of AP (44%).

Middle compartment LI was significantly influenced by age and risk for CC. Control subjects in the older age groups (50-69 years and 70-79 years) had significantly greater middle compartment LI (29% and 42% of the cells in the middle compartment had PCNA label, respectively) when compared to the younger age groups (age 20-29 and 30-49 groups had 13% and 18% of the total cells in the middle compartment with PCNA label, respectively). Among control subjects who had no history of colonic disease, there was a linear increase in the PCNA middle LI with increasing age ($r = 0.88$, $P< 0.0001$, data not shown). All subjects with a history of colonic disease had significantly greater middle LI than subjects with no history of colonic diseases.

Top compartment LI was significantly greater in subjects with FAP when compared to all other groups. Top compartment LI was greater than 6% in subjects with FAP, but was less than 1% for subjects in all other age and risk categories. Among controls, there was a linear increase in PCNA top LI with increasing age ($P < 0.05$).

Although total crypt Ki-67 LI was not influenced by age or colonic disease, we were interested in the overall patterns of Ki-67 protein expression among these groups. Ki-67 labeling was observed primarily in the basal compartment with some labeling in the middle compartment and very little in the top compartment (Table 5). Ki-67 basal compartment labeling index was significantly influenced by age and risk for CC. Control subjects in the younger age groups (20-29 years and 30-49 years) had significantly

greater basal LI (21% and 23% of the total cells in the basal compartment had Ki-67 label) when compared to the subjects in the oldest age group (70-79 years [16%]). Among control subjects, there was a linear decrease in Ki-67 basal LI with increasing age ($P < 0.05$).

When comparing all age and disease groups, there were no overall significant differences detected in Ki-67 LI in the middle and top compartments of the colonic crypts. However, we did observe a linear increase in Ki-67 middle compartment LI with increasing age ($P<0.0001$) when only control subjects with no history of colonic disease were compared.

Crypt height

Crypt height (Table 3) was significantly influenced by age and risk category. Control subjects in the oldest (70-79 years) and youngest (20-29 years) age categories had significantly taller crypts (average of 60 and 57 cells per hemicrypt, respectively) than subjects in the intermediate age groups. Subjects with a history of AP had significantly taller (65 cells per hemicrypt) colonic crypts than all subjects with no history of colonic diseases.

Relative PCNA levels

PCNA protein levels quantified by the slot-blot method were significantly affected by age and risk for CC (Table 6). Among control subjects, PCNA content increased with increasing age. Among control subjects having no history of colonic disease, elderly subjects (70-79 years of age) had approximately 2.5 times as much

PCNA protein per 20μg mucosal protein as the youngest subjects (20-29 years of age). The middle aged groups (30-49 years and 50-69 years) had intermediate PCNA levels.

Subjects with a history of adenomatous polyps, FAP and CC had approximately twice as much PCNA protein per 20μg mucosal protein as the oldest subjects with no history of colonic disease (Table 6). Among the risk categories (subjects with AP, FAP, and CC), subjects with a history of CC had the greatest expression of PCNA in the colonic mucosa.

Correlation among biomarkers (IHC indices of Ki-67 and PCNA and Relative PCNA levels)

Ki-67 total crypt LI was positively correlated with Ki-67 PZ ($r = 0.47$, $P = 0.0009$), but was not significantly correlated to any indices of PCNA expression (Table 7). Ki-67 PZ was significantly correlated with PCNA total crypt LI, PCNA middle crypt LI and PCNA PZ, and tended to be correlated with quantified PCNA. PCNA total crypt LI, PCNA PZ, PCNA middle crypt LI and relative PCNA protein quantified by slot-blot analysis all were highly correlated with each other (individual values, $r = 0.56 – 0.94$, $P<0.0001$).

E. DISCUSSION

In this study we assessed and compared the proliferative indices of colonic cells in normal colonic mucosa of subjects at varying age and risk for CC with the use of PCNA and Ki-67 using traditional immunohistochemistry methods. To the best of our knowledge, no one has reported PCNA and Ki-67 expression in normal colonic mucosa

of subjects at varying ages. An expansion of the proliferative zone as measured by both PCNA and Ki-67 was observed with increasing age in normal subjects, and also was observed in subjects, regardless of age, who had a history of colonic diseases (adenomatous polyps, FAP and CC). We also demonstrated that relative PCNA levels in colonic epithelium biopsies determined by a simple immunoblot method is a useful indicator of mucosal proliferative activity in the colon. Quantified PCNA levels, PCNA total LI, PCNA PZ and PCNA middle compartment LI of all subjects correlated with each other and showed a linear increase with increasing age among subjects having no history of colonic disease.

We observed that both total crypt PCNA LI and PZ increased with age in control subjects and also increased considerably in subjects who had a history of colonic disease (Table 3). Since there is a high incidence of CC in aged population, the relationship between changes in colonic cell proliferation and aging has been the focus of other studies. Researchers have reported that aging is characterized by an overall increase of epithelial cell proliferation in colorectal mucosa and by an upward expansion of the proliferative compartment, similar to that observed in a population at risk for colon cancer. The contribution of age as a risk factor for CC as measured by proliferation indices was first examined by Deschner et al., in 1988. They report a significant increase (1.3 times) in the LI (using tritiated thymidine) in normal mucosa of rectal biopsies of older control groups (age range of 33-78 years, mean age of 55.9 ±14.3) when compared to younger control groups (age range of 22-27, mean age of 23.3 ± 1.5). Increases in cell proliferation in colorectal mucosa of senescent rats have also been shown by other techniques in animals (Holt and Yeh, 1988; Lee et al., 2000; Xiao et al., 2000; Lee et al.,

2001). Roncucci et al. (1988) used tritiated thymidine autoradiography to demonstrate an overall increase in rectal epithelial cell proliferation and an expansion of the proliferative compartment with age in subjects in three age groups of 30 to 51, 51 to 65 and 66 to 90 years. In their study they reported significant differences in total labeling index and top compartment labeling index in normal rectal mucosa of people of 66 years and higher (12.9% and 0.04%) when compared to the youngest age group of 30 to 50 years of age (9.5 % and 0.01%). More recently, Sjöqvist et al. (2002) using DNA-flow cytometry methods demonstrated that the S-phase fraction of colonic epithelial cells (biopsies obtained from right, mid and left colon) increased linearly with age of normal subjects (age range of 21-80). They reported that the younger age group (mean of 41± 8.9 years) had a statistically lower S-phase fraction of 2.37 ± 1.38% when compared to older age group (mean of 69 ± 8.4 years) with S-phase fraction of 2.91 ± 1.66%. In our study, total crypt PCNA LI was affected by age with the highest indices (29% - 30%) observed in the older age groups (age range of 50-79 years, mean age of 65.8 ± 3.9 years). The total crypt PCNA LI was 1.4 times greater than that observed in subjects in the younger age groups (age range of 20-49 years, mean age range of 30.8 ± 4.9 years). The differences between these age groups reported in our study with PCNA is comparable to the increases (1.3) reported by Deschner et al. (1988) and Roncucci et al. (1988) using another proliferation marker, tritiated thymidine in similar age range of control subjects. Several studies have also demonstrated that PCNA correlates well with other proliferation markers (e.g. BrdU) in both normal and neoplastic colonic epithelium in humans (Kubota and Kino, 1995; Tanaka et al., 1995; Yoshikawa and Utsunomiya, 1996; Shpitz et al., 1997; McShane et al., 1998; Yerly-Motta et al., 1999; Wu et al., 2000; Díez et al., 2003).

Many studies have used IHC methods to demonstrate increased PCNA labeling indices in neoplastic tissues of adenomatous polyps and colon tumors (Yoshikawa and Utsunomiya, 1996; Bostick et al., 1997a; Shpitz et al., 1997; Ottaviani et al., 1999; Saito and Mori, 1999; Wu et al., 2000; Díez et al., 2003). In particular, PCNA expression (as measured by immunohistochemistry) has been used in human studies to demonstrate a step-wise increase in proliferation indices during neoplastic progression of colonic lesions (Shpitz et al., 1997), for predicting the potential for metastases in invasive colorectal carcinoma (Tanaka et al., 1995; Choi et al., 1997), and to predict the development of metachronous adenomas (Ottaviani et al., 1999). Studies also have demonstrated increased PCNA labeling indices along with increases in growth factors (such as EGF receptor kinase and TGF-α) (Malecka-Panas et al., 1997; Barnes et al., 1998) and overexpression of cyclins-(such as Cyclin D) (Izawa et al., 2002) in colonic tissue. In our study the overall total crypt PCNA LI also was significantly influenced by disease conditions with the greatest indices (39%) observed in normal appearing mucosa of subjects with FAP when compared to normal mucosa of subjects with a history of CC (36%) and subjects with a history of adenomatous polyps (34%) (Table 3).The mean total crypt PCNA LI measured in normal colonic mucosa of our study subjects with colonic diseases (mean age of 48.7± 11.6 years) were statistically higher than the measurements observed in subjects in the oldest age group (age range of 70-79 years, mean age of 72.1 ± 1.9 years). Moreover, in the FAP group, the subjects had an age range of 9 to 46 years with mean age of 23.6 ± 13.5 years. All subjects with FAP had statistically comparable total crypt LI with a mean of 40 % ± 1%. These findings clearly demonstrate the effect of neoplasia on colonic proliferation in normal mucosa regardless of age in these subjects.

Our PCNA IHC results also were consistent with findings from other studies (Bromely et al., 1996; Bostick et al., 1997b; Malecka-Panas et al., 1997) on colonic proliferation in normal-appearing colonic mucosa of subjects with adenomas, FAP and CC when compared to controls in similar age range. Our observed increases in total crypt PCNA LI also suggest there is a delay in epithelial cell differentiation with increasing age and risk for CC

Normally, proliferation in colonic crypts is restricted to the basal 60% of the crypts. As colonocytes migrate up the crypt, they undergo terminal differentiation. Ultimately, after approximately 7 days of transit time to the crypt surface, colonocytes undergo programmed cell death and are extruded. Under normal circumstances, cell proliferation does not occur in the upper 40% of the crypts (Lipkin, 1971). An expansion of the proliferative compartment toward the crypt surface (hyperproliferation in the middle and upper thirds of the crypt) is considered a preneoplastic biomarker (Lipkin, 1988) and has been associated with increased risk for development of adenomatous polyps and carcinoma (Risio et al., 1991). Expansion of the proliferation zone has been shown in a variety of neoplastic conditions, most notably in FAP and in colon tumorigenesis (Lipkin, 1984; Lipkin et al., 1984; Rozen et al., 1991; Rozen, 1992; Richter et al., 1993; Rooney et al., 1993; Mills et al., 1995; Sträter et al., 1995). In general, expansion of the proliferative compartment indicates that normal mechanisms controlling colonic epithelial cell proliferation and differentiation are compromised. In our study an expansion of the proliferative zone as measured by both PCNA and Ki-67 was observed with increasing age in normal subjects with the greatest expansion (56 % and 41%, respectively) observed in the oldest subjects (mean age 72.1± 1.9 years). This is

approximately an increase of 1.5 times in proliferative zone (with both PCNA and Ki-67) when compared to subjects in the youngest age group (20-29 years with a mean age of 22.7 ± 3.0 years). The mid and top compartmental indices of PCNA also significantly increased with age (Table 4). The significant differences we observed in the PCNA middle compartment LI among the groups further confirm that an upward shift in the proliferative compartment occurred with increasing age and risk for CC. The pattern of changes we observed in Ki-67 PZ among the groups was generally similar to that observed for PCNA. However, among older normal subjects or those with AP, CC, or FAP, PCNA PZ expanded to a greater extent than that of Ki-67. This observation strongly suggests that PCNA PZ increased both as a consequence of expanded zone of cell proliferation (indicated by expanded Ki-67 PZ) and due to delayed terminal differentiation of colonic epithelial cells with increasing age and risk for CC. Therefore, it is important to have age matched subjects in studies of colon cell proliferation. Future experiments should also more carefully examine mechanisms controlling colonic epithelial cell differentiation and factors that cause delayed differentiation in older persons and those who have other risk factors associated with colon cancer development.

The total crypt Ki-67 LI did not show any age or disease effects, however the mid compartment LI as measured by Ki-67 was significantly affected by age with the highest (7%) observed in the oldest age subjects (Table 5). An expansion of the zone of Ki-67 labeling was observed in older normal subjects and those with disease conditions. Our Ki-67 labeling results are consistent with those observed by other groups. While some researchers have shown that Ki-67 LI can be used as a valuable prognostic indicator in cancers including CC (Gerdes et al., 1991; Tungekar et al., 1991; Wintzer et al., 1991;

Suzuki et al., 1992; Kang et al., 1997; Akedo et al., 2001; Garrity et al., 2004), others (Sahin et al., 1994; Green et al., 1998; Paspatis et al., 1998) have demonstrated no differences in Ki-67 total labeling indices when comparing normal subjects with persons with CC or high risk for developing CC. However Paspatis et al. (1998) and Mills et al. (1995) reported an expansion of proliferative compartment in subjects with neoplasms and in subjects with FAP respectively despite no differences in the overall total labeling indices with Ki-67 which is also consistent with findings reported in our study. Ki-67 is a cell proliferation marker that is primarily expressed during the S-phase of cell cycle and absent in Go while PCNA accumulates as the cells progress through G_1, S, and M phases of the cell cycle and returns to low levels during G_0. Terminally differentiated cells do not express PCNA. While Ki-67 labeling provides an estimate of total cell proliferation in tissues (Bruno and Darzynkiewicz, 1992; Zacchetti et al., 2003), unlike PCNA, it does not necessarily reflect a cell's capacity to undergo another round of cell division (Prelich et al., 1987; Toschi and Bravo, 1988; Zacchetti et al., 2003). Other studies comparing expression of both PCNA and Ki-67 have demonstrated consistently higher PCNA indices, especially in colorectal and breast tumors (McCormick et al., 1993; Gasparini et al., 1994; Silvestrini, 1994; Yerly-Motta et al., 1999; Le Pessot et al., 2001). The probable reason for this discordance is variability in expression of both antigens during the cell cycle, with PCNA expression being more persistent (half-life of 20 hrs, Bravo and MacDonald-Bravo, 1987; Toschi and Bravo, 1988; Scott et al., 1991) throughout the cell cycle than that of Ki-67 (half-life 1-1.5 hours, Du Manoir et al., 1991; Gerdes et al., 1991; Scott et al., 1991; Bruno and Darzynkiewicz, 1992).

The cell concentration of PCNA is directly correlated with the proliferative state of the cell (Prelich et al., 1987). The presence of PCNA in G_0 -phase cells would indicate that these cells maintain the capacity to go through another round of cell division. Conversely, the absence of PCNA is indicative that a cell has undergone terminal differentiation (Bromely et al., 1996). Hence, loss of PCNA expression appears to be potentially useful as a differentiation marker of colonic epithelial cells. Furthermore, detection and quantitation of PCNA content can represent proliferative status in the colonic crypts.

In this study we also demonstrated that quantitation of PCNA content in colonic epithelium biopsies by a relatively simple immunoblotting method was a useful indicator of mucosal proliferative activity in the colon. This approach was less laborious and tedious than the immunohistochemical methods used to measure cell cycle-associated antigens as indicators of mucosal proliferative activity in the colon. To our knowledge, no other groups have used quantitative analysis of PCNA protein in normal mucosa of subjects to measure colonic mucosal proliferation. Izawa et al. (2002) used Western blotting to measure Cyclin D_1 and PCNA expression in colonic polyps of humans. However, they did not compare expression of these proteins in colonic polyps with expression of the same proteins in normal-appearing mucosa in their subjects, nor did they compare their results with subjects who had no history of colonic disease. The slot-blot method used in our study to assess PCNA has an advantage versus western blotting methods of being able to screen dozens of samples simultaneously. A disadvantage of the slot-blot approach versus immunohistochemical methods is that information on location of PCNA expression is not measurable with this approach. However, we found that

PCNA protein slot-blotting results were highly correlated with PCNA proliferative zone as well as total crypt and middle compartment labeling indices. We believe that this approach would be particularly well-suited to the analysis of colonic biopsies from large-scale clinical trials for studying diet or drug effects on colon cancer risk.

As illustrated in Table 6, relative PCNA levels significantly increased with age of normal subjects and also were dramatically increased in subjects with colonic disease (history of adenomatous polyps, FAP and CC). Among the control subjects, we observed that elderly subjects (age range of 70-79 years, mean age of 72.1 ± 1.9 years) had approximately 2.5 times as much relative PCNA levels in the colonic mucosa as the youngest subjects (age range of 20-29 years, mean age of 22.7 ± 3.0 years). We also found elevated expression of total PCNA protein in colonic mucosa of subjects with adenomatous polyps (mean age of 57.8 ± 12.4 years), familial adenomatous polyposis (mean age of 23.6 ± 13.4 years) and colon cancer (mean age of 65.7 ± 9.0 years). Regardless of age, the greatest relative PCNA protein levels were observed in subjects with a history of CC, with a 1.4 fold increase when compared to subjects with a history of FAP and with a 1.6 fold increase when compared to subjects with adenomatous polyps. These results indicate that the relative PCNA levels in human colonic mucosa accurately reflect proliferative activity in colonic epithelial cells as evidenced by the PCNA proliferation indices. In addition, relative PCNA levels, PCNA total labeling index, PCNA proliferation zone and PCNA middle compartment labeling index of all subjects correlated with each other and showed a linear increase with increasing age among subjects having no history of colonic disease (Table 7). Furthermore, elevated expression of total PCNA protein in colonic mucosa of subjects with adenomatous polyps, familial

adenomatous polyposis and colon cancer, as well as in older subjects who had no history of colonic disease, indicates that the relative PCNA protein levels can be a useful indicator of colon cancer risk. Taken together, the differences in relative PCNA levels as well as with PCNA proliferation indices reported in our study seems to be consistent with proliferation patterns observed in studies using other proliferation markers in colonic mucosa of people at risk for CC.

F. CONCLUSION

There is a need for development of colon cancer intermediate end points or precursors that can be measured easily, that would require relatively small samples and which could be used in short intervention trials. This would be an important advance in understanding colon cancer etiology and assessing interventions to reduce colon cancer risk. In our study, the relative levels of PCNA in endoscopically normal appearing colon mucosa were affected by age, history of adenomatous polyps, personal history of CC and FAP. Although there were no significant differences in Ki-67 total LI, PCNA total LI, PZ, and Ki-67 PZ differed significantly with age and risk for CC. These correlate with the differences observed in the relative PCNA levels among the groups. The slot-blot technique and subsequent use of densitometry to quantitate PCNA levels in colonic biopsy samples was easy and reproducible. The relative PCNA levels in conjunction with proliferative compartment analyses could serve to identify groups of high risk population for CC. In addition this could be used as a sensitive index to study dietary modulations on colon carcinogenesis.

Table 3. Crypt heights[1] and Proliferation indices – PCNA and Ki-67 LI[2] & PZ[3] in colon of subjects at varying age and risk for colon cancer

Categories[5]	Proliferation Indices[4]				CH
	PCNA LI	Ki-67 LI	PCNA PZ	Ki-67 PZ	
20-29yrs (6)	$0.203^{a} \pm 0.013$	0.081 ± 0.006	$0.375^{a} \pm 0.024$	$0.276^{a} \pm 0.026$	$57^{bc} \pm 2$
30-49yrs (6)	$0.233^{a} \pm 0.013$	0.089 ± 0.006	$0.406^{a} \pm 0.024$	$0.309^{ab} \pm 0.026$	$52^{a} \pm 2$
50-69yrs (6)	$0.292^{b} \pm 0.013$	0.083 ± 0.006	$0.492^{b} \pm 0.024$	$0.353^{bc} \pm 0.026$	$53^{ab} \pm 1$
70-79yrs (7)	$0.301^{b} \pm 0.012$	0.079 ± 0.006	$0.562^{c} \pm 0.022$	$0.411^{c} \pm 0.024$	$60^{cd} \pm 1$
AP (8)	$0.344^{c} \pm 0.011$	0.075 ± 0.005	$0.553^{bc} \pm 0.021$	$0.379^{c} \pm 0.022$	$65^{e} \pm 1$
FAP (7)	$0.398^{d} \pm 0.012$	0.078 ± 0.006	$0.633^{d} \pm 0.022$	$0.380^{c} \pm 0.024$	$63^{de} \pm 1$
CC (6)	$0.360^{c} \pm 0.013$	0.081 ± 0.006	$0.548^{bc} \pm 0.024$	$0.357^{bc} \pm 0.026$	$63^{de} \pm 2$

[1] CH – Crypt Height, total number of cells in the hemicrypt from the base to the mouth of the crypt

[2] LI – Total Crypt Labeling Index, obtained by dividing the number of labeled cells in a hemicrypt by crypt height.

[3] PZ – Proliferation Zone, obtained by dividing the position of the highest labeled cell from the hemicrypt base by the crypt height

[4] Values are presented as means ± SEM. Means in the same column not sharing a common superscript are different ($P<0.05$).

[5] Age groups are subjects who have had no history of adenomas or colon cancer or FAP
AP – People with a history of adenomatous polyps
FAP - People with a history of familial adenomatous polyposis
CC - People with a history of colon cancer

Table 4. PCNA compartmental labeling indices (for basal, middle and top crypt compartments) in colon of subjects at varying age and risk for colon cancer

Categories[3]	PCNA Indices[1,2]		
	Basal LI	Mid LI	Top LI
20-29yrs (6)	0.481^{ab} ±0.025	0.129^{a} ±0.026	0.001^{a} ±0.013
30-49yrs (6)	0.507^{bc} ±0.025	0.182^{a} ±0.026	0.000^{a} ±0.013
50-69yrs (6)	0.578^{d} ±0.025	0.294^{b} ±0.026	0.000^{a} ±0.013
70-79yrs (7)	0.432^{a} ±0.023	0.416^{c} ±0.024	0.009^{a} ±0.012
AP (8)	0.442^{ab} ±0.021	0.530^{d} ±0.023	0.000^{a} ±0.011
FAP (7)	0.574^{cd} ±0.023	0.554^{d} ±0.024	0.066^{b} ±0.012
CC (6)	0.582^{d} ±0.025	0.492^{d} ±0.026	0.006^{a} ±0.013

[1] Values are presented as means ± SEM. Means in the same column not sharing a common superscript are different ($P<0.05$).

[2] Basal LI– Labeling Index in Basal one-third compartment of the crypt;
Mid LI – Labeling Index in Middle one-third compartment of the crypt;
Top LI – Labeling Index in Top one-third compartment of the crypt;

[3] Age groups are subjects who have had no history of adenomas or colon cancer or FAP
AP – People with a history of adenomatous polyps
FAP - People with a history of familial adenomatous polyposis
CC - People with a history of colon cancer

Table 5. Ki-67 compartmental labeling indices (for basal, middle and top crypt compartments) in colon of subjects at varying age and risk for colon cancer.

Categories[3]	Ki-67 Indices[1, 2]		
	Basal LI	Mid LI	Top LI
20-29yrs (6)	0.214^{bc} ±0.012	0.027 ±0.011	0.000 ±0.000
30-49yrs (6)	0.228^{c} ±0.012	0.039 ±0.011	0.000 ±0.000
50-69yrs (6)	0.188^{ab} ±0.012	0.061 ±0.011	0.000 ±0.000
70-79yrs (7)	0.161^{a} ±0.011	0.073 ±0.011	0.002 ±0.000
AP (8)	0.161^{a} ±0.010	0.064 ±0.010	0.000 ±0.000
FAP (7)	0.166^{a} ±0.011	0.068 ±0.010	0.000 ±0.000
CC (6)	0.183^{ab} ±0.012	0.059 ±0.011	0.000 ±0.000

[1] Values are presented as means ± SEM. Means in the same column not sharing a common superscript are different ($P<0.05$).

[2] Basal LI– Labeling Index in Basal one-third compartment of the crypt;
Mid LI – Labeling Index in Middle one-third compartment of the crypt;
Top LI – Labeling Index in Top one-third compartment of the crypt;

[3] Age groups are subjects who have had no history of adenomas or colon cancer or FAP
AP – People with a history of adenomatous polyps
FAP - People with a history of familial adenomatous polyposis
CC - People with a history of colon cancer

Table 6. Relative PCNA levels in colonic mucosa of subjects at varying age and risk for colon cancer

Categories[1]	N	Relative PCNA[2]
1: 20-29yrs	6	$304^{a} \pm 59$
2: 30-49yrs	6	$614^{bc} \pm 59$
3: 50-69yrs	6	$550^{b} \pm 59$
4: 70-79yrs	7	$763^{c} \pm 55$
5: AP	8	$1251^{d} \pm 51$
6: FAP	7	$1432^{e} \pm 55$
7: CC	6	$2047^{f} \pm 59$

[1] Age groups are subjects who have had no history of adenomas or colon cancer or FAP
AP – People with a history of adenomatous polyps
FAP - People with a history of familial adenomatous polyposis
CC - People with a history of colon cancer

[2] Values (these are relative values based on densitometry per 20 μg mucosal protein) are presented as means ± SEM. Means in the same column not sharing a common superscript are different ($P<0.05$).

Table 7. Pearson correlation coefficients for Ki-67 & PCNA LI (total crypt LI) and PZ, PCNA middle LI and relative PCNA levels in colonic mucosa of subjects at varying age and risk for colon cancer.

Indices	Ki-67 LI	Ki-67 PZ	PCNA LI	PCNA PZ	Relative PCNA
Ki-67 LI	X				
Ki-67 PZ	0.47 P = 0.0009	X			
PCNA LI	- 0.12 NS[1]	0.42 P = 0.0041	X		
PCNA PZ	- 0.10 NS[1]	0.49 P = 0.0006	0.90 P < 0.0001	X	
Relative PCNA	- 0.09 NS[1]	0.25 P = 0.0942	0.73 P < 0.0001	0.56 P < 0.0001	X
PCNA Middle LI	- 0.16 NS[1]	0.48 P = 0.0007	0.94 P < 0.0001	0.90 P < 0.0001	0.72 P < 0.0001

[1] Not significant

CHAPTER IV. BIOMARKERS FOR COLON CANCER RISK BEFORE AND AFTER CONSUMING SOY OR CASEIN SUPPLEMENTS FOR ONE YEAR

A. ABSTRACT

Increased cell proliferation is a common feature of the promotion stage of colon carcinogenesis. Mucosal cell proliferation before and after dietary treatment or other interventions is frequently used to monitor treatment effectiveness. A double-blind, prospective study consisting of forty two subjects (34-70 yrs of age having a history of adenomatous polyps or colon cancer) was designed to see if consumption of one of two supplements containing 38 g/d of soy protein isolate (containing 58 mg total genistein) or supplement containing 40 g/d of calcium caseinate for one year would influence colonic epithelial cell proliferation as measured by PCNA and Ki-67 labeling in and PCNA protein expression in colonic epithelial cells. The two soy supplements differed only in their calcium contents. Four colonic biopsies from each subject were obtained before and after supplementation and used for immunohistochemical detection of PCNA and Ki-67 antigens in colon crypts and determination of relative PCNA protein expression by slot-blotting. There was a statistically significant reduction in both PCNA labeling index (LI) (beginning versus ending values - soy: 30% and 26% $P < 0.05$; casein: 28% no change) and PCNA proliferation zone (PZ) (beginning versus ending values - soy: 53% and 45% $P < 0.05$; casein: 49% and 48%) during the course of the experiment by subjects that consumed soy supplement but not in subjects who consumed casein. There were no significant treatment effects on the total Ki-67 LI (9%) and Ki-67 PZ (46%). The relative expression of PCNA protein in colonic mucosa was significantly ($P < 0.05$) reduced by consumption of either of the soy supplements, but was not influenced by consumption of

the casein supplement. Since the presence of PCNA indicates the cells capacity to undergo cell division, the decrease in PCNA LI and PZ denotes a lowering in the proliferating capacity of colonic crypts in those subjects that consumed the soy supplement. This observation indicates that consumption of soy protein isolate supplements caused changes in colonic epithelial cell proliferation patterns that are consistent with a reduced risk for developing colon cancer.

B. INTRODUCTION

Colorectal cancer (CC) is the third most common cancer and second most common cause of mortality in the U.S. (American Cancer Society, 2005). People with sporadic adenomatous polyps are known to have significantly greater risk for developing CC compared to the general population (Luebeck and Moolgavkar, 2002). The presence of proliferating epithelial cells in the upper 40% of colon crypts is considered a good criteria for assessing high risk for CC (Lipkin, 1987). An upward expansion of the zone of cell proliferation in colonic epithelium occurs in individuals with sporadic adenomas of the large intestine (Deschner, 1985; Lipkin, 1987; Wilson et al., 1990; Risio et al., 1991). Therefore, patients with sporadic colon adenomas represent a suitable population for studying if consumption of certain foods will cause a downward shift in the proliferating compartment of colon crypts and thus, reduce risk of CC.

The role of diet in the etiology of CC has been recognized and extensively studied in the last few decades (Potter, 1996; Lipkin et al., 1999; Sanderson et al., 2004). A review by American Institute for Cancer Research (AICR/WCRF, 1997) estimates that that 67-75% of CC incidence could be prevented by adequate diets. Epidemiological

studies have established a positive correlation between colon cancer risk and increased intake of animal fat (Wynder et al., 1969; Reddy et al., 1978; McKeown-Eyssen and Bright-See, 1984; Sandler, 1996) and an inverse association between colon cancer risk and consumption of diets rich in fruits and vegetables (Steinmetz and Potter, 1991; Thun et al., 1992; Potter, 1996). Increasing consumption of foods that contain anti-carcinogenic phytochemicals (e.g. fruits, vegetables, soybeans) is a major public health strategy for reducing the incidence of many diet-related cancers such as CC. Soybean foods in particular have been promoted as having the potential to reduce CC risk (Messina and Bennink, 1998). Soy contains a number of compounds having potential anti-cancer activity. These compounds include isoflavones, protease inhibitors, phytosterols, saponins and phytate.

Soybeans are a rich and unique source of the isoflavones genistein and daidzein (1-3 mg of total isoflavone per gram of dry matter). Isoflavones typically occur as glycosides and are normally associated with the protein fraction of soybeans. The type and nature of processing can affect the quantity of isoflavones in processed soy products. A portion of the isoflavones are lost during preparation of SPI, but certain conventional means of preparation (with alkaline solubilization and acid precipitation) can produce SPI which contain 1mg or more total isoflavones per gram of dry matter (Lucas and Riaz, 1995).

Numerous rodent studies have examined the potential of dietary soy to reduce colon cancer development. Hakkak et al. (2001) reported a statistically significant reduction ($P<0.05$) in AOM-induced CC when male rats were fed soy protein isolate (SPI) compared to casein. Bennink (2001) demonstrated significant inhibition of colon

cancer development by feeding full fat soy flour or defatted soy flour to AOM-induced rats. However, some animal studies reported that soybean protein had no protective effect against colon carcinogenesis when compared to beef protein (Reddy et al., 1976; Clinton et al., 1979; McIntosh et al., 1995).

The potential of soy isoflavones to reduce cancer risk has been the focus of many recent studies (Sorensen et al., 1998; Gee et al., 2000; Guo et al., 2004). Genistein has received particular interest because of its ability to inhibit several enzymes (particularly tyrosine kinases, (Akiyama et al., 1987) involved in cell cycle regulation. Numerous *in vitro* studies have demonstrated that genistein inhibits the growth of a variety of cancer cell lines at concentrations ranging from 5–50 μmol/liter (Yanagihara et al., 1993; Booth et al., 1999; Wenzel et al., 2000). Several studies have examined the efficacy of isoflavones to reduce colon cancer risk in animal models. Studies conducted by Pereira et al. (1994) found that addition of 150 mg genistein per kg of diets fed to rats resulted in 34 % reduction in the numbers of aberrant crypt foci (ACF) in rat colons after initiating CC by azoxymethane (AOM) injection. Helms and Gallaher (1995) found that feeding genistein (370 mg/kg diet) reduced DMH-induced ACF by 35%. Collectively, these studies suggest that feeding purified isoflavones inhibit preneoplastic biomarkers of colon carcinogenesis. Increased labeling index (LI) and an upward shift in the proliferation zone (PZ) within a colonic crypt are two biomarkers believed to be related to increased risk for developing CC (Lipkin, 1987; Deschner et al., 1988; Gerdes et al., 1993; Mills et al., 1995; Akedo et al., 2001). A demonstrated reduction in LI and PZ by dietary soy would further substantiate its potential efficacy to reduce CC risk.

Another factor that has been extensively studied for its potential to reduce CC risk is dietary calcium. The association between calcium intake and colorectal remains uncertain, despite considerable epidemiologic and laboratory research (Baron et al., 1999). It has been proposed that dietary calcium binds to bile acids in the lumen of the bowel, inhibiting their potential to promote colon cancer (Newmark et al., 1984; Van der Meer and De Vries, 1985; Van der Meer et al., 1990). Rodent studies examining the influence of dietary calcium on colon carcinogenesis have reported inhibition of bile-induced mucosal damage and experimental carcinogenesis (Govers et al., 1994; Piard et al., 1994). Several randomized human studies have found that supplementary calcium (range of 1.25 g to 2 g/day) reduces rectal mucosal proliferation (Lipkin and Newmark, 1985; Rozen et al., 1989; Bostick et al., 1995; Wargovich et al., 1995a), although other human studies have not found this effect (Stern et al., 1990; Armitage et al., 1995; Baron et al., 1995; Cats et al., 1995; Karagas et al., 1998).

While there have been a number of studies examining the influence of dietary calcium on biomarkers of cell proliferation in colonic epithelium in animal models and in humans, we are aware of no studies examining the effect of soy protein on colonic epithelial cell proliferation in humans. The objective of this research was to determine if consuming 38 g/day of soy protein isolate supplement (containing 58 mg total genistein) would reduce CC risk as reflected by changes in colonic epithelial cell proliferation patterns in humans. A secondary objective was to determine if adding calcium to the soy protein isolate supplement would alter its impact on colon epithelial cell proliferation.

C. MATERIALS AND METHODS

Experimental Design

This investigation was a 12-month, randomized, double-blind study with parallel treatment and control groups evaluating the effects of soy protein on colonic epithelial cell proliferation and other potential parameters of colon cancer risk. Subjects were randomly assigned either to one of two supplements in the treatment arm (soy protein isolate with or without added calcium) or the control arm (calcium caseinate). Treatment assignments were determined by a statistician. Changes in colon epithelial cell proliferation patterns were measured before and after consuming the supplements for one year. This research was approved by the Michigan State University Committee for Research Involving Human Subjects and informed, written consent was obtained from all study participants.

Subjects

Subjects were eligible for participation in this study if they had a previous or current history of two or more colonic adenomatous polyps or history of colon cancer and if they were between the ages of 35-70 years at study entry. Potential subjects were excluded if they had a history of allergy to soy or soy products, b) milk allergy, c) serious intestinal disease such as chronic inflammatory bowel disease, or d) significant diseases of the cardiovascular, pulmonary, hepatic, gastrointestinal, renal, neurological, hematological systems. Subjects were primarily recruited from a list of patients from the gastroenterology unit at the Clinical Center of Michigan State University. Some subjects were solicited from the greater Lansing area by newspaper advertisement.

Advertisements were placed at nearby hospitals and clinics to encourage recruitment. Potential subjects (n = 1, 200) having a history of colon polyps were screened for eligibility. Of this group, 400 subjects met the eligibility criteria. After verification of previous history of adenomatous polyps or CC from patient records, seventy subjects entered the study. Patient histories, physical examinations, flexible sigmoidoscopy or colonoscopy, colon biopsies and blood samples were obtained for baseline information. After consuming the supplements for twelve months, blood samples and colon biopsies were obtained. Forty-two subjects (27 males and 15 females) completed the study.

Subjects were less than 70 years old (age range: 35-70 years), were ambulatory, did not have any other gastro-intestinal disorders, consumed 2 or fewer aspirins/day, and were requested to not consume soy products. Subjects provided bimonthly urine samples to allow monitoring of urinary isoflavone excretion to confirm supplement consumption (by the groups consuming either of the two soy protein isolate-based supplements) and to confirm minimal soy consumption by the group consuming the casein-based supplement. At baseline and at the end of one year, each participant also completed a food frequency questionnaire.

Dietary Treatments

Age- and gender-matched subjects were randomly assigned to one of the three treatments: (a) casein beverage [n = 13 subjects completed the study, mean age = 58, 8 males and 5 females] (b) soy protein isolate (SPI) beverage [n = 16, mean age = 59, 13 males and 3 females], and (c) SPI beverage without calcium [n = 13, mean age = 59, 6 males and 7 females]. The SPI-based supplements were based on Supro^{TM} brand soy

protein manufactured by Protein Technologies International (Saint Louis, MO). The SPI beverage powder was commercially available and contained 0.6 g calcium per packet. Given the potential effects of calcium on indices of colon epithelial cell proliferation to be measured in the study, a soy treatment lacking calcium was formulated to allow direct determination of the effect of soy protein without additional supplemental calcium. Calcium caseinate was the protein source used in the casein-based supplement. Hence, the casein-based supplement powder contained 1.01 g calcium per packet. Since the supplements were formulated to provide nutrients other than protein, they also contained additional vitamins (A, B_1, B_2, niacin, B_6, C, D and B_{12}) and minerals (magnesium and zinc). The supplements were formulated to contain equivalent concentrations of all essential nutrients except calcium. The nutrient composition for each packet (27 g) of each of the three dietary supplements is presented in Table 8. Subjects were instructed to consume 2 packets/day of each supplement. In addition to other nutrients, the supplements each provided approximately 40 g of protein and 200 kcal per day. The two SPI-based supplements provided approximately 58 mg of genistein (primarily as glycosides) daily. As a supplement to their normal diets, subjects self-administered these beverage powder supplements which they consumed twice each day (morning and evening) for one year. Subjects were provided instructions on how to incorporate the beverage powder into their typical diet. Typically, the beverage powder supplements were mixed either in water, juice or carbonated beverages prior to consumption. There was no other dietary intervention imposed on these free-living subjects.

Nutrient intake

Nutrient intake was calculated from food frequency questionnaires (FFQ) administered at baseline and after consumption of treatment or control beverage for one year (Health Habits and History Questionnaire developed by Dr. Gladys Block, National Cancer Institute, DIETSYS-Version 3.0).

Compliance

Subject compliance with the experimental protocol was assessed by: 1) self-completed consumption log; 2) monitoring unused supplement; 3) phone contacts and 4) by measuring isoflavone excretion by HPLC analysis of bi-monthly urine samples.

Tissue procurement and processing

Colon mucosa biopsies were obtained from each subject at the beginning of the study prior to starting dietary treatment. Another set of biopsies was obtained from each subject at the completion of the one-year study period. Subjects received an oral electrolyte bowel preparation (Golytely^{TM} containing polyethylene glycol 3350^{TM}, NaCl, KCl, $NaHCO_3$, and Na_2SO_4) the evening before flexible sigmoidoscopy or colonoscopy. Colonic biopsy samples (4 per subject) were obtained during flexible sigmoidoscopy or colonoscopy using "jumbo" forceps. Biopsies (average size 3 X 8 mm) were obtained from normal appearing mucosa 30-40 cms from the anal verge. All biopsies were immediately rinsed in phosphate buffered saline (pH 7.4). Two biopsies were placed individually in 0.6 ml microcentrifuge tubes and immediately frozen on dry ice for subsequent slot-blot quantification of PCNA. The remaining two biopsies were pinned

flat to cardboard strips and fixed in B_5 fixative for 2 hours, then fixed in neutral buffered formalin for 2–6 hours. The fixed biopsies were placed between foam pads in histopath cassettes, dehydrated, infiltrated with paraffin and embedded with paraffin using standard histologic procedures.

PCNA and Ki-67 IHC analyses

Three micron thick sections of paraffin-embedded biopsy samples were cut and mounted on poly-L-lysine coated glass slides and dried at 58° C for 2 hours. Sections were cleared of paraffin with xylene, hydrated in increasing ratios of water:ethanol, and treated with iodine:potassium iodide and 5% sodium thiosulfate to remove mercury. Samples were then subjected to antigen retrieval (10 mMol/L citrate buffer, pH 6.0, 95 °C) for 20 minutes before immersion in 0.3% hydrogen peroxide for 10 minutes to quench endogenous peroxide activity. Following rinses in tris-buffered saline (TBS), sections were then incubated with primary antibody (1:100 dilution of PC10 [anti-PCNA] or MM1 [anti- Ki-67] respectively in 1% BSA in Tris buffered saline, pH 7.4) overnight at 4 °C. After subsequent incubations in biotinylated rabbit anti-mouse immunoglobulins (diluted 1:100, 45 min), followed by peroxidase-conjugated streptavidin (diluted 1:300, 45 min), they were treated with 3-amino-9-ethylcarbazole (AEC) and color development was monitored for 10–20 minutes until red staining was evident. The sections were rinsed twice with 10 mMol/L TBS in between incubations with antibodies, streptavidin and AEC. Slides were then counter-stained with hemotoxylin and cover-slipped using Faramount mounting medium. Appropriate positive (human tonsil) and negative controls (omission of primary antibody) were used for evaluation of PCNA and Ki-67 staining.

The primary antibodies were obtained from Novacastra (now Vector Laboratories; Burlingame, CA), and biotinylated rabbit anti-mouse immunoglobulin, streptavidin, AEC, and Faramount were obtained from Dako (Carpinteria, CA).

Quantification of PCNA and Ki-67 Immunohistochemistry

PCNA and Ki-67 labeling were quantified by the percentage and positions of positively stained (red) nuclei within full-length crypts in colonic sections. Well-oriented longitudinally-sectioned full-length crypts (n = 10 per subject) were identified by light microscopy. A scorable crypt was defined as one in which the base touched the muscularis mucosa and had an open lumen at the top. The number and position of PCNA and Ki-67 labeled cells and crypt heights were recorded for ten full crypts for each subject to determine cell proliferation parameters. All cells with a red nuclear staining were counted as positive. The center-most cell at the base of the crypt, and in between the hemicrypts, was designated as cell number one and the cell at the top of the crypt mouth was considered the highest cell. A continuous column of cells from cell number one to the highest cell were counted along each hemicrypt wall of each crypt. Crypt height was defined as the number of cells per hemicrypt.

Total Crypt Labeling Index (LI) was calculated by dividing the number of labeled cells in a hemicrypt by the total number of cells in a hemicrypt (crypt height). These values were averaged for at least 20 hemicrypts (representing 10 full-length crypts) to obtain the LI for each subject. Compartment Labeling Indexes (basal LI, mid LI and top LI) were calculated by dividing the number of labeled cells within each third (basal, middle and top) of each hemicrypt by the number of cells in each third of the respective

hemicrypt. Proliferation zone (PZ) was obtained by dividing the position of the highest labeled cell from the hemicrypt base by the total number of cells in the hemicrypt. Each of these PZ values were averaged for at least 20 hemicrypts to obtain the overall PZ for each subject.

PCNA Measurement by Slot-blot

Slot-blot analysis to determine relative PCNA protein concentration was conducted on frozen biopsies obtained at the beginning and end of treatment. Biopsies were homogenized with a glass pestle using 0.5ml of non-denaturing buffer containing protease inhibitors (20mMol/L MOPS, 150 mMol/L sodium chloride, 1% vol/vol Triton X-100, 1% wt/vol deoxycholate, 0.1% wt/vol SDS, 1 mMol/L EDTA, 100 μMol/L sodium orthovandate, 250 μMol/L PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin). Total protein concentrations in homogenates were determined by the biocinchoninic assay using BSA as the standard. Relative PCNA levels in tissue homogenates were then determined by immuno-quantification using slot-blot methodology. PVDF membranes (Immobilon P membranes; Millipore, Billerica, MA) were wetted in methanol and then transferred to PBS. Twenty μg of protein from each sample (dilutions in PBS) was vacuum blotted on to the PVDF membranes using a slot-blot apparatus (Hoefer Scientific instruments). The membranes were held overnight at 4 °C in blocking buffer (3% BSA in TBS with Tween 20). The membranes were then incubated overnight with monoclonal PC-10 antibody (1:1000 dilution of primary antibody in blocking buffer). After rinsing with wash buffer (TBS with Tween 20) the membranes were incubated in biotinylated secondary antibody (1:1000 dilution of goat anti-mouse IgG in wash buffer for one hour).

Blots were rinsed twice with wash buffer and then incubated with streptavidin-peroxidase for 1 hour. After subsequent rinses with wash buffer, the membranes were treated with a chemiluminescence reagent (Dupont NEN research products) and light emission was captured by autoradiography. Developed autoradiography films were quantified by scanning densitometry. Known amounts of mouse IgG Fc fragments (standard curve ranging from 0.3125ng to 10.00ng) were included in each blot to allow for normalization of staining intensity across blots.

Statistical Analysis

Crypt height, PCNA and Ki-67 proliferation indices (total crypt LI, PZ, and compartmental LI for basal, middle, and top compartments of the crypts) and relative PCNA quantified by the slot-blot method each were statistically analyzed by two-way Analysis of Variance (treatment and time as the two factors) using the General Linear Models procedure of SAS (version 6.11, SAS Institute Inc., Cary, NC). When significant treatment or time effects were detected ($P < 0.05$), the least significant difference was used to compare treatment means. The means and standard errors presented in Tables 4, 5 and 6 were generated from this 2-way analysis of variance. Comparisons of beginning (T_B) and ending (T_E) values within each treatment and parameter were conducted using paired t-tests for each time pair.

D. RESULTS

Nutrient Intake from Food Frequency Records

Table 10 provides information on the intakes of selected nutrients, based on the food frequency questionnaire administered at the beginning and end of the supplement period, of the 42 participants who completed the study. There were no statistically significant differences among the three groups for any of the dietary variables presented except for calcium intake in subjects (Table 10). The total calcium intake (both from diet, multi-vitamin use and beverage supplement) varied across the treatment groups, with the calcium intake in the casein beverage supplement groups being the highest when compared to the soy beverage supplement groups.

Whole Crypt Proliferation Indices

Results for whole crypt PCNA and Ki-67 labeling indices are presented in Table 11. Crypt height was not influenced by time (beginning versus end) or by dietary treatments and, for all subjects, averaged 64 cells per hemicrypt. Prior to initiation of the dietary treatments (T_B values), approximately 30% (PCNA) and 9% (Ki-67) of the colonic crypt cells stained positively for PCNA and Ki-67, respectively. Total crypt LI for PCNA and Ki-67 were not significantly different among the supplement groups at the beginning of the study. Subjects who consumed the SPI with calcium supplement had a significant ($P < 0.05$) reduction in total crypt PCNA LI from 30.3% initially to 23.6% after one year of supplementation. There was no statistically significant reduction in the mean total crypt PCNA LI in subjects that consumed the SPI without added calcium or the casein supplements, although subjects consuming the SPI minus calcium supplement

tended ($P < 0.10$) to have reduced whole crypt PCNA LI at the end of the study (28.5%) as compared to the beginning of the treatment period (31.5%). When the two soy-containing treatments were combined, there was a significant ($P < 0.05$) reduction in total crypt PCNA LI after one year of supplement consumption. Total crypt labeling with Ki-67 was not influenced by supplement or time.

Prior to initiation of the dietary treatments, (T_B values), PCNA and Ki-67 PZ values did not differ among the treatment groups and averaged (across all treatments) 51% and 45% for PCNA and Ki-67, respectively (Table 11). Subjects who consumed the SPI with calcium supplement had a significant ($P < 0.05$) reduction in the mean PCNA PZ from 55% initially to 44% after one year. There was no statistically significant reduction in the mean total crypt PCNA PZ in subjects that consumed the SPI without calcium (beginning value: 49% and ending value: 47%) or the casein (beginning value: 49% and ending value: 47%) supplements. When the two SPI supplement (+/- calcium) groups were combined, there was a statistically significant reduction in the mean PCNA PZ from 53% to 45% during the course of the study. The mean total crypt Ki-67 PZ was not influenced by dietary supplement or by time.

Compartmental Labeling Indices (basal LI, mid LI, top LI)

Results for compartmental LI indices for PCNA are presented in Table 12. Given the observed changes in total crypt PCNA LI and PZ (Table 12), we were interested in determining how PCNA labeling patterns in specific regions of the crypts were influenced by consumption of soy protein. In subjects that consumed the SPI plus calcium supplement, there were significant ($P < 0.05$) reductions in PCNA LI values in

the middle compartment (32% to 19%) and top compartment (4% to 0.5%), and a tendency ($P < 0.10$) for reduced PCNA LI in the basal compartment (55% to 51%), during the course of this study. Subjects that consumed the SPI without calcium supplement also tended ($P < 0.10$) to have reduced basal compartment PCNA LI at the end of the study (55%) as compared to the beginning value (57%). There was a statistically significant increase in the basal compartment PCNA LI values (beginning value: 52% and ending value: 57%) in subjects that consumed casein beverage powder but no changes were observed in PCNA LI in the middle or top compartments for subjects consuming the casein supplement.

When the two SPI supplement (+/- calcium) groups were combined, there were significant ($P < 0.05$) reductions of PCNA LI in the basal compartment (56% to 53%), middle compartment (34% to 24%) and top compartment 3% to 0.4% during the course of the study (Table 12).

Relative PCNA Protein Concentration in Colonic Mucosa

Data on PCNA protein expression in the colonic mucosa of subjects are presented in Table 13. The values for PCNA protein are relative values based on scanning densitometry and normalized to a known concentration of mouse IgG Fc fragments. PCNA protein expression did not differ among the supplement groups at the beginning of the study. After one year of supplement consumption, there were significant ($P < 0.05$) reductions in relative PCNA protein concentrations in subjects who consumed either SPI plus calcium (10.5% reduction) or SPI minus calcium (8.3% reduction). Averaged across both soy supplement groups, there was a 9.5% reduction in relative PCNA protein

expression in colonic mucosal cells during the course of the study. Relative PCNA protein concentration in subjects consuming the casein supplement were not significantly different after supplementation when compared to the values before initiation of the study.

E. DISCUSSION

Most human studies on soy consumption and cancer risk have focused on epidemiological associations, pharmacokinetic properties of soy components, and on the effects of isoflavones on circulating hormone levels. To date, there has been no intervention trial conducted in humans to study the potential efficacy of soy or soy-containing foods to reduce risk of colon cancer. In this study, we evaluated the potential efficacy of soy protein isolate to reduce intermediate biomarkers (PCNA and Ki-67 expression in colonic crypts) associated with colorectal cancer risk in humans.

PCNA is a cell proliferation marker that accumulates as the cells progress through G_1, S, and M phases of the cell cycle and returns to low levels during G_0. Terminally differentiated cells do not express PCNA (Bromely et al., 1996). Studies have shown that PCNA can be used as a reliable marker for the proliferative activity in colonic crypts (Malecka-Panas et al., 1997; Barnes et al., 1998; Izawa et al., 2002). Many studies have used IHC methods to demonstrate increased PCNA labeling indices in neoplastic tissues of adenomatous polyps and colon tumors (Yoshikawa and Utsunomiya, 1996; Bostick, 1997; Bostick et al., 1997a; Bostick et al., 1997b; Shpitz et al., 1997; Ottaviani et al., 1999; Saito and Mori, 1999; Wu et al., 2000; Díez et al., 2003). In particular, PCNA expression (as measured by immunohistochemistry) has been used in human studies to

demonstrate a step-wise increase in proliferation indices during neoplastic progression of colonic lesions (Shpitz et al., 1997), for predicting the potential for metastases in invasive colorectal carcinoma (Tanaka et al., 1995; Choi et al., 1997), and to predict the development of metachronous adenomas (Ottaviani et al., 1999).

Ki-67 is a cell proliferation marker that is primarily expressed during the S-phase of cell cycle and is absent in G_0. While some researchers have shown that Ki-67 LI can be used as a valuable prognostic indicator in cancers including CC (Gerdes et al., 1987; Tungekar et al., 1991; Wintzer et al., 1991; Suzuki et al., 1992; Kang et al., 1997; Akedo et al., 2001; Garrity et al., 2004), others (Sahin et al., 1994; Green et al., 1998; Paspatis et al., 1998) have demonstrated no differences in Ki-67 total labeling indices when comparing normal subjects with persons with CC or high risk for developing CC. However Paspatis et al. (1998) and Mills et al. (1995) reported an expansion of proliferative compartment in subjects with neoplasms and in subjects with FAP respectively despite no differences in the overall total labeling indices with Ki-67.

Normally, proliferation in colonic crypts is restricted to the basal 60% of the crypts. As colonocytes migrate up the crypt, these cells undergo terminal differentiation. Ultimately, after approximately 7 days of transit time to the crypt surface, colonocytes undergo programmed cell death and are extruded. Under normal circumstances, cell proliferation does not occur in the upper 40% of the crypts (Lipkin, 1971). An expansion of the proliferative compartment toward the crypt surface (hyperproliferation in the middle and upper thirds of the crypt) is considered a preneoplastic biomarker (Lipkin, 1988) and has been associated with increased risk for development of adenomatous polyps and carcinoma (Risio et al., 1991). Expansion of the proliferation zone has been

shown in a variety of neoplastic conditions, most notably in FAP and in colon tumorigenesis (Rozen et al., 1991; Richter et al., 1993; Mills et al., 1995). In general, expansion of the proliferative compartment indicates that normal mechanisms controlling colonic epithelial cell proliferation and differentiation are compromised.

In our study we used the immunohistochemical detection of PCNA and Ki-67 to detect crypt cell proliferation patterns in the biopsies of subjects that consumed soy or casein beverage for one year. The subjects participating in this study either had a history of adenomatous polyps or colon cancer and, therefore, represented a group that was at high risk for colon cancer. We have previously demonstrated (Chapter 3) that humans who have a history of adenomatous polyps or colon cancer, when compared to age-matched normal subjects, have increased PCNA LI and increased relative expression of PCNA protein in colonic crypts. Our overall objective was to determine if consumption of soy protein supplement with isoflavones would reduce these indices of colon cancer risk.

We observed a significant decrease in total crypt PCNA labeling index and proliferation zone in colonic mucosal biopsies of subjects that consumed the soy protein supplements for one year. Subjects who consumed the casein supplement did not have any changes in PCNA or Ki-67 labeling indices in colonic mucosa. It is noteworthy that no significant effects of supplement consumption on Ki-67 LI or PZ were detected, whereas soy supplement consumption reduced whole crypt PCNA LI and PZ (Table 11). Taken together, these observations suggest that consumption of the soy supplement did not influence the S-phase fraction of colonic epithelial cells, but did reduce the fraction of cells capable of undergoing another round of cell division (based on reduced PCNA LI).

Further, reduction of the PCNA PZ by soy supplementation suggests that colonic epithelial cells underwent terminal cell differentiation relatively earlier than in subjects consuming the casein-based supplement. Overall, these observations indicate that consumption of the soy protein supplement is associated with changes in intermediate biomarkers of colonic epithelial cell proliferation that are consistent with reduced risk for colon cancer development.

Our findings are that consumption of SPI consumption reduced colonic epithelial cell proliferation and, presumably, CC risk. Studies in laboratory animals have shown that a soy-rich diet inhibits chemically induced carcinogenesis (Pereira et al., 1994; Koratkar and Rao, 1997; Kennedy et al., 2002). Hakkak et al. (2001) found that feeding rats a SPI-based diet reduced incidence of AOM-induced colonic tumors when compared with rats fed a casein-based diet. Rats fed the casein diet had a 50% incidence of colon tumors compared with 12% on soy protein based diet (Hakkak et al., 2001). Guo et al. (2004) reported that feeding an isolate of soy protein containing isoflavones reduced colon tumor incidence in ovariectomized estrogen receptor-α knockout female mice relative to that observed in these mice when fed a casein-based diet.

The precise mechanisms whereby SPI prevents colon cancer in animal models are unknown. However, factors that are associated with the isolated protein, particularly the isoflavones genistein and daidzein, have been implicated as being important (Messina and Erdman, 1998). Several potential mechanisms have been proposed for the anti-carcinogenic activity of isoflavones. These proposed mechanisms include a) inhibition of tyrosine kinases; b) inhibition of proteases; c) anti-oxidant activity, d) increased synthesis and decreased degradation of steroid hormone binding globulin; e) weak estrogen

agonist/antagonist activity mediated through estrogen receptor α; f) altered hormone metabolism or action; g) induction of Phase I and II detoxification systems; and h) inhibition of carcinogen activation (Wattenberg, 1985; Barnes et al., 1990; Ronis et al., 1999). Several studies have indicated that treatment with purified isoflavones or soy-based diets can induce phase II enzymes such as glutathione S-transferases (GSTs) in the liver, small intestine and colon (Appelt and Reicks, 1999).

In addition to isoflavones, soy contains a number of other biologically active factors, including saponins, phytosterols, protease inhibitors and inositol hexaphosphate, that have been postulated to influence colon carcinogenesis (Messina and Bennink, 1998). Given the composition of SPI used in this study, we are unable to ascertain the relative contributions of isoflavones or the other constituents present in soy to our observed reductions in colonic epithelial cell proliferation. We have previously reported that addition of 150 mg genistein/kg of a diet based on soy concentrate led to significant reductions in ACF numbers and PCNA LI and PZ in colonic mucosa of AOM-initiated rats (Chapter 5). These animal model data suggest that isoflavones alone could be responsible for the effects on intermediate biomarkers observed in this study.

A secondary objective of this study was to assess the impact of supplemental calcium on intermediate biomarkers for CC risk. There is some evidence that calcium prevents colorectal carcinogenesis by influencing a complex series of signaling events induced at various tiers of colonic cell organization (Lamprecht and Lipkin, 2001). Several animal studies (Lipkin et al., 1999) and some human studies (Lipkin and Newmark, 1985; Rozen et al., 1989; Wargovich et al., 1992; Bostick et al., 1995; Holt et al., 1998) but not all (Gregoire et al., 1989; Stern et al., 1990; Kleibeuker et al., 1993;

Baron et al., 1995) show that consumption of calcium and dairy foods reduced colonic epithelial cell proliferation. Few clinical trials also have suggested that calcium intake reduced the recurrence of colorectal adenomas (Baron et al., 1999; Bonithon-Kopp et al., 2000). Overall, the benefical effects of calcium on CC seems modest and contradictory.

The casein supplement used in this study contained the calcium salt of casein as the protein source, and therefore, subjects consuming this supplement were receiving an additional 2 g of calcium per day. When compared with subjects who consumed either of the soy-based supplements, subjects consuming the casein supplement also reported greater consumption of calcium from mineral supplements during the end-of-study FFQ. Despite the greater intake of calcium by subjects consuming the casein treatment, no significant reductions in total crypt PCNA LI or PZ or relative PCNA protein concentration were observed in colon biopsies obtained from these persons at the end of this study. The PCNA proliferation indices of subjects assigned the casein beverage treatment at the beginning of the study were numerically lower than those of the subjects assigned to either SPI treatment, but these differences in initial PCNA LI and PZ were not statistically different among treatments. Thus, we do not believe that the lack of effect of calcium caseinate on colonic mucosal proliferation indices can be explained by slight differences in initial PCNA LI or PZ. Moreover, other studies (Bostick et al., 1993a; Bostick et al., 1993b; Baron et al., 1995; Weisgerber et al., 1996; Alberts et al., 1997) have also found no effect of calcium supplementation on colonic epithelial cell proliferation contrary to what was previously demonstrated (Lipkin and Newmark, 1985; Wargovich and Lointier, 1987; Rozen et al., 1989; Wargovich et al., 1992). The original studies of Lipkin and Newmark (1985) and associates all reported significant but modest

reductions in colonic proliferation indices in subjects when calcium was provided in doses of 1.2-1.5 g/day. However in a recent review article Chia (2004) concluded that that there is strong evidence of a threshold effect and that calcium intake by humans greater than 700mg/day does not benefit the colon.

We did observe that subjects consuming SPI plus calcium had greater reductions in colonic mucosal PCNA LI and PZ when compared to subjects consuming SPI without calcium. Thus, we can not rule out the possibility that effects of SPI and calcium on colonic epithelial cell proliferation may be additive.

In a previous study (Chapter 3) we provided evidence indicating that PCNA proliferation indices may be a better discriminator than Ki-67 for predicting colon cancer risk. Colon cancer has long been recognized as an age-related disease (American Cancer Society, 1998) and research by Roncucci et al. (1988) and Deschner et al. (1988) has consistently demonstrated an increase in colonic epithelial cell proliferation indices with increasing age. We previously measured PCNA labeling index and proliferation zone in human subjects that had known intestinal diseases (CC, FAP, adenomatous polyps) and in subjects of varying age with no history of colonic disease. We found that both PCNA LI and PZ increased incrementally with increasing age (from 20 to 70 years of age) in normal subjects. Persons having a history of adenomatous polyps had PCNA LI and PZ values that were comparable to or greater than that observed in normal subjects ranging in age from 70-79 years (Chapter 3). The beginning proliferation indices for subjects consuming the SPI plus calcium supplement (Table 11) in this study were comparable to indices observed in subjects in the 70-79 year age group or in subjects with a history of adenomatous polyps in our previous study (Chapter 3). The end-of-study PCNA LI and

PZ indices of subjects that consumed SPI in this study compares favorably to that observed in 50-year-old normal subjects having no history of colonic diseases reported in our earlier study (Chapter 3). The American Cancer Society (2005) reports that the percentage of people developing malignant colon cancer at the age range of 55 – 60 years is much lower than that for seventy-five years of age. Therefore, we interpret the downward shift in proliferation zone and decrease in labeling index after consumption of soy protein isolate as a meaningful decrease in colon cancer risk.

We previously demonstrated that relative PCNA protein concentrations (as determined by slot-blot analysis) were highly correlated with PCNA LI and PZ indices in colonic crypts of humans who varied in age and risk for CC development (Chapter 3). In this study, we further demonstrated that relative PCNA protein concentrations responded to dietary supplementation with SPI. This indicates that, like whole crypt PCNA LI and PZ, relative PCNA protein concentration in the colonic mucosa is responsive to dietary intervention. Further study is necessary to determine if relative PCNA protein expression in colonic mucosa is a potentially valid method to predict CC risk of humans that is more facile than whole-crypt PCNA LI and PZ analyses.

In summary, we demonstrated in this study that supplementing the diets of free-living adults who were at elevated risk for CC with SPI caused significant changes in PCNA indices that correlate with reduced risk for CC. Administration of a calcium caseinate supplement did not influence whole-crypt PCNA LI or PZ despite a much greater intake of calcium. Given that SPI supplementation reduced PCNA LI and PZ and total PCNA protein concentrations, but did not influence Ki-67 LI or PZ, we conclude

that the major effect of SPI supplementation on colonic epithelial cells was the induction of more rapid cell differentiation as these cells migrate toward the crypt surface.

Table 8. Nutrient Composition of Dietary Supplements

Intake/ supplement packet[1]	Dietary Beverage Supplement		
	Casein	SPI[2] plus Calcium	SPI minus Calcium
Moisture (g)	0.72	0.63	0.80
Protein (g)	20.49	18.76	18.95
Fat (g)	0.25	1.10	1.10
Ash (g)	1.83	2.78	1.10
CHO (g, by difference)	4.21	4.28	4.00
Kcal	101	102	102
Calcium (g)	1.01	0.60	0.05
Magnesium (mg)	53	55	53
Zinc (mg)	3.46	2.41	2.04
Vitamin A (IU)	952	1060	880
Vitamin D (IU)	139	132	130
Vitamin C (mg)	3.16	2.70	3.15
Vitamin B_1 (mg)	0.16	0.13	0.22
Vitamin B_2 (mg)	0.36	0.45	0.54
Niacin (mg)	0.29	0.40	0.44
Vitamin B_6 (mg)	0.21	0.22	0.29
Folacin (μg)	19	74	45
Vitamin B_{12} (μg)	1.24	2.34	1.29

[1] Two packets were consumed per day.
[2] SPI – Soy Protein Isolate.

Table 9. Characteristics of Study Participants at Baseline

Characteristics at Baseline	Dietary Beverage Supplement		
	Casein	SPI[1] plus Calcium	SPI minus Calcium
	(n = 13)	(n = 16)	(n = 13)
Male Gender (%)	62	81	46
Mean Age (yrs)	58	59	59
Family/Personal history of colorectal carcinoma (number)	1/13	0	2/13

[1] SPI – Soy Protein Isolate.

Table 10. Daily Intakes of Selected Dietary Nutrients by Study Participants at the beginning (T_B) and ending (T_E) of each treatment

	Dietary Beverage Supplement					
	Casein		SPI[1] plus Calcium		SPI minus Calcium	
	(n = 13)		(n = 16)		(n = 13)	
Selected Dietary Intakes[3]	T_B[2]	T_E	T_B	T_E	T_B	T_E
Calories (kcal/day)	1705	1641	1934	1654	1651	1747
Protein (g/day)	64	62	75	66	64	76
Total Fat (g/day)	79	68	78	62	71	98
Carbohydrates (g/day)	177	187	219	206	186	212
Calcium from food (mg/day)	752	705	770	882	701	763
Calcium from mineral supplement (mg/day)	125	409	535	96	50	90
Calcium from beverage treatment (mg/day)	-	2000	-	1200	-	100
Use of multi-vitamin mineral pill (%)	17	36	21	15	10	9

[1] SPI – Soy Protein Isolate

[2] T_B – Indices prior to consumption of beverage treatment, and T_E – Indices after consumption of beverage treatment for one year

[3] Values represent pooled results after consumption of beverage supplement at the end of the one-year study period.

Table 11. Whole Colonic Crypt Proliferation Indices[1, 7] (PCNA and Ki-67) of subjects

	Dietary Beverage Supplement			
Indices	**Casein** (n = 13; mean age=58)	**SPI[2] plus Calcium** (n = 16; mean age =59)	**SPI minus Calcium** (n = 13; mean age=59)	**SPI +/- Calcium[3]** (n = 29)
Crypt Height (Pooled value)[4]	62.0 ± 1.4	64.1 ± 1.3	65.8 ± 1.4	
PCNA LI[5]-T_B[1]	0.286 ± 0.013	0.303 ± 0.012	0.315 ± 0.013	0.308 ± 0.010
T_E[1]	0.282 ± 0.013	0.236* ± 0.012	0.285† ± 0.013	0.258* ± 0.010
PZ[6]- T_B	0.487 ± 0.024	0.554 ± 0.022	0.498 ± 0.024	0.529 ± 0.017
T_E	0.476 ± 0.024	0.436* ± 0.022	0.473 ± 0.024	0.453* ± 0.017
Ki-67 LI- T_B	0.093 ± 0.005	0.097 ± 0.005	0.095 ± 0.005	
T_E	0.090 ± 0.005	0.095 ± 0.005	0.088 ± 0.005	
PZ- T_B	0.460 ± 0.019	0.456 ± 0.017	0.460 ± 0.018	
T_E	0.450 ± 0.019	0.460 ± 0.017	0.465 ± 0.018	

[1] Whole colonic crypt proliferation Indices were determined before (T_B) and after (T_E) consumption of beverage supplements for one year.

[2] SPI – Soy Protein Isolate.

[3] Pooled values after combining soy beverage treatments +/- calcium.

[4] Crypt Height – total number of cells in the hemicrypt from the base to the mouth of the crypt prior to consumption of the dietary treatment. The values presented in this table are averaged for the T_B and T_E time points within each treatment because no significant effects of time or treatment were detected.

[5] LI – Total Crypt Labeling Index, obtained by dividing the number of labeled cells in a hemicrypt by crypt height.

[6] PZ – Proliferation Zone, obtained by dividing the position of the highest labeled cell from the hemicrypt base by the crypt height.

[7] Values are presented as means ± SEM.
Within a treatment and parameter, an asterisk (*) indicates a significant difference ($P < 0.05$) between T_E vs. T_B values. A cross (†) indicates a trend ($P < 0.10$) for a difference between T_E vs. T_B values.

Table 12. PCNA compartmental proliferation indices[1,7] in colonic crypts of subjects

	Dietary Beverage Supplement			
PCNA Indices	**Casein** (n = 13; mean age=58)	**SPI[2] plus Calcium** (n = 16; mean age =59)	**SPI minus Calcium** (n = 13; mean age=59)	**SPI +/- Calcium[3]** (n = 29)
Basal LI[4]- T_B[1]	0.523 ± 0.014	0.549 ± 0.013	0.574 ± 0.014	0.560 ± 0.010
T_E[1]	0.574* ± 0.014	0.509† ± 0.013	0.553† ± 0.014	0.529* ± 0.010
Mid LI[5]- T_B	0.318 ± 0.035	0.318 ± 0.031	0.360 ± 0.035	0.337 ± 0.024
T_E	0.252 ± 0.035	0.192* ± 0.031	0.298 ± 0.035	0.239* ± 0.024
Top LI[6]- T_B	0.015 ± 0.009	0.041 ± 0.008	0.012 ± 0.009	0.028 ± 0.006
T_E	0.021 ± 0.009	0.005* ± 0.008	0.004 ± 0.009	0.004* ± 0.006

[1] PCNA compartmental proliferation indices in colonic crypts were determined before (T_B) and after (T_E) consumption of beverage supplements for one year.
[2] SPI – Soy Protein Isolate.
[3] Pooled values after combining soy beverage treatments ± calcium.
[4] Basal LI– Labeling Index in Basal ⅓rd compartment of the crypt.
[5] Mid LI – Labeling Index in Middle ⅓rd compartment of the crypt.
[6] Top LI – Labeling Index in Top ⅓rd compartment of the crypt.
[7] Values are presented as means ± SEM.
Within a treatment and parameter, an asterisk (*) indicates a significant difference ($P < 0.05$) between T_E vs. T_B values. A cross (†) indicates a trend ($P < 0.10$) for a difference between T_E vs. T_B values.

Table 13. Effect of Casein, SPI[1] plus Calcium and SPI minus Calcium on the mean beginning and ending relative PCNA (slot-blot methodology) protein levels[4] in colonic mucosa

Indices	Casein (n = 13)	SPI plus Calcium (n = 16)	SPI minus Calcium (n = 13)	SPI +/- Calcium[2] (n = 29)
T_B[3]	1215 ± 37	1223 ± 33	1230 ± 37	1226 ± 24
T_E[3]	1200 ± 37	1095* ± 33	1128* ± 37	1110* ± 24

[1] SPI – Soy Protein Isolate.

[2] Pooled values after combining soy beverage treatments ± calcium.

[3] PCNA protein expression in colonic mucosa was determined before (T_B) and after (T_E) consumption of beverage supplements for one year.

[4] Values (these are relative values based on densitometry per 20 μg mucosal protein) are presented as means ± SEM.
An asterisk (*) indicates a significant change ($P < 0.05$) for T_E vs. T_B values.

CHAPTER V. EFFECT OF SOY FLAKES, SOY FLOUR, SOY CONCENTRATE, GENISTEIN AND CALCIUM ON ABERRANT CRYPT FOCI DEVELOPMENT AND COLONIC EPITHELIAL CELL PROLIFERATION IN RATS INJECTED WITH AOM

A. ABSTRACT

Increased colonic epithelial cell proliferation and delayed terminal cell differentiation are commonly associated with increased risk for colon carcinogenesis. The objective of this study was to investigate the chemopreventive potential of different soy products against colon carcinogenesis in carcinogen-treated rats as reflected by changes in aberrant crypt foci (ACF) development and colonic epithelial cell proliferation. Colon cancer was initiated in rats by injecting 15 mg/kg BW AOM subcutaneously at 21 and 28 days of age. At 35 days of age, rats were placed on the dietary treatments (n = 15/treatment). The dietary treatments contained soy with differing degrees of processing: whole soy known as full fat soy flakes (SFK), soy with fat soluble components removed (de-fatted soy flour, [SFL]) and soy concentrate which has the fat soluble and aqueous-alcohol soluble components removed (soy concentrate [SC]). Other dietary treatments included soy concentrate plus 150 mg/kg genistein (GEN), and soy concentrate plus 5 g/kg calcium (CAL). Rats were fed these diets for twelve weeks, at which point they were sacrificed, the entire colons removed for aberrant crypt foci and proliferation analyses. Rats consuming SC had the greatest ($P < 0.05$) numbers of total ACF, large ACF and total aberrant crypts among all treatment groups. Supplementation of the soy concentrate diet with either genistein or calcium resulted in the lowest ($P < 0.05$) numbers of ACF, large ACF and total aberrant crypts. Cell proliferation was assessed by PCNA and Ki-67 labeling index (LI) and proliferative zone (PZ), and

quantified PCNA levels in colon mucosa. Rats fed SFK had the lowest ($P < 0.05$) PCNA LI and PZ (0.180, 0.342) and these indices were significantly lower than those observed in rats consuming SC (0.336, 0.509). The PCNA LI and PZ of rats consuming SFL (0.203, 0.348), CAL (0.215, 0.388) or GEN (0.222, 0.389) were not statistically different from each other but were significantly lower than that observed in the SC-fed rats. Rats consuming SC also had the greatest ($P < 0.05$) Ki-67 LI and PZ values among all treatment groups. Quantified PCNA protein levels (by slot-blot methodology) in colonic epithelium of rats fed SFL or SFK were significantly lower than that observed in rats consuming SC, GEN, or CAL diets. All biomarkers of cell proliferation tested in this study (PCNA and Ki-67 LI and PZ, quantified PCNA protein) were positively correlated ($P < 0.05$) with each other and with numbers of ACF. Overall, these results indicate that one or more aqueous-alcohol soluble but not fat soluble phytochemicals present in soy is responsible for the reduction in ACF and reduced colonic epithelial cell proliferation observed in AOM-treated rats. Furthermore, the protective effects of soy flakes and soy flour against AOM-induced ACF development were replicated by addition of 150 mg/kg genistein or 5 g/kg calcium to the SC diet.

B. INTRODUCTION

Colon cancer (CC) is a serious health problem in most developed countries and is the second most common cause of cancer mortality in the United States (American Cancer Society, 2005). Asian populations have relatively low rates of breast, prostate, and CC when compared with Western cultures. Asian diets are typically lower in total and saturated fat and higher in dietary fiber than Western diets. Another significant

dietary difference between Asian and Western population is the consumption of soy-containing foods. Published reviews of epidemiologic literature, by McKeown-Eyseen and Bright-See (1984), Messina et al. (1994), Messina and Bennink (1998), and Spector et al. (2003) have all examined the relationship between soy food intake and colorectal cancer. The reviewers observed that very few of the ecological and case-control studies reported a statistically significant reduction in CC risk with soy consumption. Therefore, it is safe to conclude, that while there is no compelling epidemiologic evidence to support an inverse relationship between soy intake and risk of colorectal cancer, there exist a need for studies that are designed specifically to determine if soy consumption lowers the risk for CC.

Soybeans and soybean-based foods are a good source of genistein. Genistein and other soy phytochemicals such as phytosterols, saponins, protease inhibitors and phytate have been reported to have potential anticancer properties. Several studies (Shamsuddin et al., 1989; Pretlow et al., 1992b; Pereira et al., 1994; Awad et al., 1997; Koratkar and Rao, 1997; Kennedy et al., 2002) have demonstrated that feeding isolated soy phytochemicals in large amounts reduces experimental CC in animals. In a series of studies conducted by Bennink (2001) it was reported that diets containing either full-fat soy flour or defatted soy flour reduced colon tumor incidence in male Fisher 344 rats, whereas soy concentrate did not reduce colon tumor incidence. More recently a study by Murillo et al. (2004) reported that supplementation with soy bean flour significantly decreased the total number of ACF in the colon of rats injected with AOM, a 53% inhibition when compared to the control group (rodent chow). However, some animal studies indicate that soy-containing diets may actually increase CC risk (Rao et al., 1997;

Gee et al., 2000). McIntosh et al. (1995) compared the effects of various protein sources (20g/100g diet) on induction of colorectal neoplasia by dimethyl hydrazine (DMH) in male Sprague Dawley rats and observed numerically higher tumor incidence in rats fed soy protein than in those fed whey or casein, but these effects were not statistically different. Davies et al. (1999) found that diets high in fat, low in calcium, and containing high-isoflavone soy protein did not protect against ACF development in AOM-treated Fisher 344 rats.

Much of the interest in genistein as a potential anti-carcinogen has been promoted by its well-established anti-proliferative effects against cancer cells *in vitro*. Studies have shown that genistein inhibits the growth of hormone-dependent cells from tumors of the breast (Peterson and Barnes, 1991) and prostate (Peterson and Barnes, 1993), and also inhibits proliferation of leukemia (Makishima et al., 1991), melanoma (Rauth et al., 1997) and gastric cancer cells (Matsukawa et al., 1993; Yanagihara et al., 1993). Genistein has been shown to block mitosis and stimulate apoptosis in colon cancer cell lines (Booth et al., 1999; Wenzel et al., 2000). These findings have led to studies in animal models that have attempted to demonstrate the anti-carcinogeic effect of purified genistein or of soy products containing isoflavones. Pereira et al. (1994) observed a protective effect of genistein (150mg/Kg diet) against induction of ACF (preneoplastic lesions), which have been shown to correlate with the subsequent development of adenomas and adenocarcinomas in rat distal colon. However, a study by Rao et al. (1997) reported that genistein (250mg/Kg diet) increased colon tumor numbers in AOM-treated rats.

Kennedy et al. (2002) reported that feeding a soybean extract containing the Bowman-Birk protease inhibitor reduced DMH induced colon carcinogenesis in mice and rats. Koratkar and Rao (1997) found that a diet containing 3% soy saponins, when administered after AOM injection, reduced the average number of ACF per colon in CF1 mice as compared to mice consuming the control diet. Investigators have examined the effect of phytosterols on colonycyte proliferation in rats (Awad et al., 1997) and report a normalization of cholic acid induced hyperproliferation of colonycytes in rats. Raicht et al. (1980) examined the growth of methylnitrosurea induced tumors in rats fed 0.2% soy phytosterols and reported a 60% reduction in tumor incidence with phytosterols. Thus, several components in soy have been reported to reduce carcinogen-induced precancerous lesions or tumors in the colon of animal models for human colon cancer.

Dysregulated epithelial cell proliferation within normal-appearing colonic mucosa is a strong predictor of increased susceptibility to neoplasia (Lipkin, 1988). Measures of colonic epithelial cell proliferation (Labeling Index and Proliferation Zone) have been proposed as indices of colorectal cancer risk, or as surrogate endpoint biomarkers for cancer chemoprevention studies (Scalmati and Lipkin, 1993; Alberts et al., 1994; Syngal et al., 2000; Kelloff et al., 2004). The transition of colonic mucosal cells from hyperproliferation to atypia/dysplasia is characterized by development of abnormal nuclear and/or cellular shapes and development of colon crypts having larger cells, raised crypt surfaces, and abnormal staining pattern. These foci of abnormal colon crypts have been termed aberrant crypt foci (ACF), which are the primary pre-cancerous lesion found in rodents treated with colon carcinogens (Bird et al., 1986).

Soy foods are one of only a few dietary sources of the isoflavone genistein. Methods of processing can dramatically influence the concentrations of isoflavones and other phytochemicals present in soy products. De-fatted soy flour (obtained by hexane solvent extraction of soy flakes) contains significant concentrations of isoflavones and other phytochemicals. However, ethanol extraction of soy flour to produce soy protein concentrate removes the majority of the isoflavones and oligosaccharides that were originally present in soy flour (U.S. Department of Agriculture, 1999).

We hypothesize that the inhibitory effects of soy-containing diets with phtochemicals against colon cancer promotion are associated with reduced colonic epithelial cell proliferation and reduced development of ACF. Therefore, the objective of this study was to determine if soy-containing diets having differing concentrations of phytochemicals (especially isoflavones) can reduce ACF and indices of colonic epithelial cell proliferation (as assessed by PCNA and Ki-67 immunohistochemistry in colonic tissue and by PCNA protein quantification by immunoblotting) in AOM-initiated rats. A further objective was to determine if changes in colon epithelial cell proliferation patterns (as influenced by dietary treatments) correlate with ACF formation in rats treated with AOM. Our final objective was to determine if dietary calcium concentration (1 or 5 g/kg of diet) would influence ACF development and markers of colonic epithelial cell proliferation.

C. MATERIALS AND METHODS

Materials

Azoxymethane was purchased from Ash-Stevens, Inc (Detroit, MI). Full fat soy flakes, defatted soy flour and soy concentrate were obtained from Central Soya (Ft Wayne, IN). Genistein was obtained from Dr. Muralee Nair at Michigan State University and was synthesized as described previously (Chang et al., 1994). Corn starch and sucrose were obtained from Michigan State University Food Stores. All other dietary ingredients were obtained from Dyets Inc. (Bethlehem, PA).

Animals

Male Fischer 344 rats were obtained at 3 weeks of age from Harlan Sprague-Dawley Inc (Indianapolis). The rats were housed in plastic cages (3 rats/cage) with wood chip bedding. The animal room was maintained at 21-22 °C and 45% ± 10% relative humidity with a 12-h light-dark cycle. Diets and water were provided ad libitum. This study was approved by the Michigan State University All-University Committee on Animal Use and Care.

Diets

All diets used in this study were based on the AIN-93G diet (Reeves et al., 1993) and the compositions of diets are listed in Table 14. The basal diet was formulated to maximize the promotion of colon tumorigenesis. The calcium content of AIN-93G diet is 5 g/kg to allow for maximum bone and teeth mineralization in rapidly growing rodents. This concentration of calcium can have an anti-cancer action in the colon as indicated by

a decrease aberrant crypt foci and colonic proliferation indices in rats that were injected with AOM (Beatty et al., 1993; Li et al., 1998; Pierre et al., 2003). Therefore, we formulated all diets except the SC+Cal diet to be low (1 g/kg) in dietary calcium so that the potential anti-cancer action of other dietary ingredients would not be masked by the dietary calcium levels present in standard AIN-93G diet. An earlier study conducted by Pereira et al. (1994) reported that 150 mg/kg of genistein added to the AIN-93G diet significantly reduced the number of aberrant crypt foci (by 34% relative to controls) in F344 rats injected with AOM. Therefore, we added genistein (150 mg/kg) to the soy concentrate diet to determine if this concentration of genistein would inhibit ACF to the same extent as the soyflour diet.

All diets were formulated to contain approximately 14.8% fat and 20% protein from the various soy preparations. The concentrations of essential nutrients (except calcium) were increased by 13% due to the increased energy density of these diets compared to the standard AIN-93G diet. Diets contained 1 g/kg calcium except for the SC+Cal diet, which contained 5 g/kg calcium. The ratios of protein, minerals (with the exception of calcium), vitamins and other essential nutrients to energy were similar to the standard AIN-93G diet (Reeves et al., 1993).

Experimental design

Each dietary treatment group consisted of 15 rats. All rats, except negative controls, were injected subcutaneously with azoxymethane (15 mg/kg body wt) at 21 and 28 d of age. Because the time of day when azoxymethane injections are made is important (Pereira et al., 1994), the injections took place between 0800 and 1000. The

negative control group received saline injections instead of azoxymethane. All rats were fed the soy concentrate diet from days 21 to 35 days of age and then were switched to their respective dietary treatments at 35 d of age, and were killed 12 wk later by overexposure to carbon dioxide followed by exsanguination.

Methods

Isoflavone Analysis

Isoflavones were extracted from the soy flakes, soy flour, and soy concentrate by mixing duplicate 1 g samples of each with 20 mL of 0.1 mol/L hydrochloric acid. Eighty milliliters of methanol was added and the mixture was sonicated for 20 min, left at room temperature for 2 h, and gravity filtered with Whatman no. 1 filter paper (Whatman, Clifton, NJ). The filtrate was centrifuged at 10,000 X g and the isoflavones in the supernate were separated and quantified by HPLC. The isoflavones were separated with a reversed-phase column (Microsorb-MV, 25 X 0.46-cm column packed with C-18 silica particles of 5-mm diameter and 100-Å pore size; Rainin Instrument Co, Woburn, MA) by using a gradient mobile phase. Solvent A was 10% methanol, 89.9% water, and 0.1% acetic acid; solvent B was 99.9% methanol and 0.1% acetic acid. The amount of solvent B was increased linearly from 20% at 0 min to 30% at 2 min and then to 70% at 30 min. The isoflavones were detected at 260nm and quantified by the external standard technique.

Pathology and histology

After rats were sacrificed, the colons were removed, cut open longitudinally, and rinsed in phosphate-buffered saline (PBS, pH 7.4). One cm of the colon (3 cm from the anal verge) was excised and pinned flat to a cardboard, fixed in B5 fixative for one hour, placed between foam pads in tissue cassettes, and processed by routine paraffin embedding and sectioning for subsequent IHC. A section of the colon (proximal) was scraped with a microscope slide to obtain mucosal cells, which were frozen immediately on dry ice and saved for slot-blot quantification of PCNA. The remaining length of the colon was fixed in 2% paraformaldehyde-PBS for 1 hour at 4°C and saved for ACF quantification.

Aberrant Crypt Foci analyses

Fixed colons were stained with methylene blue (0.1% in PBS) for 3 min as previously described (McLellan and Bird, 1988), rinsed in PBS, and stored in 0.4% formaldehyde-PBS (pH 7.4) at 4°C. AOM-induced lesions appear as darkly stained, enlarged, and slightly elevated crypts that can be distinguished from the lightly stained normal mucosa. The lesions may appear as single aberrant crypts or as foci containing two or more aberrant crypts. ACF and the number of aberrant crypts per focus were visualized by stereomicroscopy. The total number of ACF, the numbers of large ACF (i.e. ACF that contained 4 or more aberrant crypts) and the total number of aberrant crypts per rat were recorded. Foci with 10 or more aberrant crypts (too many aberrant crypts per focus to count accurately) were recorded as foci containing more than 10

aberrant crypts. In summing the total numbers of aberrant crypts per rat, we assigned a value of 10 aberrant crypts to ACF that contained ten or more aberrant crypts.

PCNA and Ki-67 IHC analyses

Immunohistochemistry analyses were conducted on a sub-set of the rats from each treatment (n = 10 rats/treatment for No AOM, soy flour, soy concentrate and soy concentrate plus genistein treatments; n = 9 rats for the soy concentrate plus calcium treatment; n = 11 rats for the soy flakes treatment). Three-micron sections of paraffin-embedded biopsy samples were cut and mounted on poly-L-lysine coated glass slides and dried at 58 °C for 2 hours. Sections were cleared of paraffin with xylene, hydrated in increasing ratios of water:ethanol, and treated with iodine:potassium iodide and 5% sodium thiosulfate to remove mercury. Samples were then subjected to antigen retrieval (10 mMol/L citrate buffer, pH 6.0, 95 °C) for 20 minutes before immersion in 0.3% hydrogen peroxide for 10 minutes to quench endogenous peroxide activity. Following rinses in tris-buffered saline (TBS), sections were then incubated with primary antibody (1:100 dilution of PC10 [anti-PCNA] or MM1 [anti- Ki-67] respectively in 1% BSA in Tris buffered saline, pH 7.4) overnight at 4° C. After subsequent incubations in biotinylated rabbit anti-mouse immunoglobulins (diluted 1:100, 45 min), followed by peroxidase-conjugated streptavidin (diluted 1:300, 45 min), sections were treated with 3-amino-9-ethylcarbazole (AEC) and color development was monitored for 10–20 minutes until red staining was evident. Slides were then counter-stained with hemotoxylin and cover-slipped using Faramount mounting medium. Appropriate positive (human tonsils) and negative controls (omission of primary antibody) were used for evaluation of PCNA

and Ki-67 staining. The antibodies were obtained from Novacastra, and biotinylated rabbit anti-mouse immunoglobulin, streptavidin, AEC, and Faramount were obtained from Dako (Carpinteria, CA). The sections were rinsed twice with 10 mMol/L TBS in between incubations with antibodies, streptavidin and AEC.

Quantification of PCNA and Ki-67 Immunohistochemistry

PCNA and Ki-67 labeling were quantified by the percentage and positions of positively stained (red) cells within full-length crypts in colonic sections. Well-oriented longitudinally-sectioned full-length crypts (n = 10 per rat) were identified by light microscopy. A scorable crypt was defined as one in which the base touched the muscularis mucosa and had an open lumen at the top. The number and position of PCNA and Ki-67 labeled cells and crypt heights were recorded for ten full crypts for each rat to determine cell proliferation parameters. All cells with a red nuclear staining were counted as positive. The center-most cell at the base of the crypt, and in between the hemicrypts, was designated as cell number one and the cell at the top of the crypt mouth was considered the highest cell. A continuous column of cells from cell number one to the highest cell were counted along each hemicrypt wall of each crypt. Crypt height was defined as the number of cells per hemicrypt.

Total Crypt Labeling Index (LI) was calculated by dividing the number of labeled cells in a hemicrypt by the total number of cells in a hemicrypt (crypt height). These values were averaged for at least 20 hemicrypts (representing 10 full-length crypts) to obtain the LI for each rat. Compartment Labeling Indexes (basal LI, mid LI and top LI) were calculated by dividing the number of labeled cells within each third (basal, middle

and top) of each hemicrypt by the number of cells in each third of the respective hemicrypt. Proliferation zone (PZ) was obtained by dividing the position of the highest labeled cell from the hemicrypt base by the total number of cells in the hemicrypt. Each of these PZ values were averaged for at least 20 hemicrypts to obtain the overall PZ for each rat.

PCNA Measurement by Slot-blot

Slot-blot analysis was conducted on a sub-set of the rats from each treatment (n = 10 rats/treatment for No AOM, soy flour, soy concentrate and soy concentrate plus genistein treatments; n = 9 rats for the soy concentrate plus calcium treatment; n = 11 rats for the soy flakes treatment). These samples were from the same rats that were used for IHC determination of PCNA and Ki-67 expression. Mucosal samples were homogenized with 0.5 ml of non-denaturing buffer containing protease inhibitors (20mMol/L MOPS, 150 mMol/L sodium chloride, 1% vol/vol Triton X-100, 1% wt/vol deoxycholate, 0.1% wt/vol SDS, 1 mMol/L EDTA, 100 μMol/L sodium orthovandate, 250 μMol/L PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin) and total protein concentrations were determined by the biocinchoninic assay using BSA as the standard. Relative PCNA levels in mucosal homogenates were then determined by immuno-quantification using slot-blot methodology. PVDF membranes (Immobilon P membranes; Millipore, Billerica, MA) were wetted in methanol and then transferred to PBS. Twenty μg of protein from each sample (dilutions in PBS) was vacuum blotted on to the PVDF membranes using a slot-blot apparatus (Hoefer Scientific instruments). The membranes were held overnight at 4 °C in blocking buffer (3% BSA in TBS with Tween 20. The membranes were then

incubated for 2 hours with monoclonal PC-10 antibody (1:1000 dilution of primary antibody in blocking buffer). After rinsing with wash buffer (TBS with Tween 20) the membranes were incubated in biotinylated secondary antibody (1:1000 dilution of goat anti-mouse IgG) in wash buffer for one hour. After subsequent rinses with wash buffer, the membranes were treated with a chemiluminescence reagent (Dupont NEN research products) and light emission was captured by autoradiography. Developed autoradiography films were quantified by scanning densitometry. Known amounts of mouse IgG Fc fragments (standard curve ranging from 0.3125ng to 10.00ng) were included in each blot to allow for normalization of staining intensity across blots.

Statistical Analysis

Aberrant crypt foci, crypt height, PCNA and Ki-67 proliferation indices (total crypt LI, PZ, and compartmental LI for basal, middle, and top compartments of the crypts) and relative PCNA quantified by the slot-blot method each were statistically analyzed as a completely randomized design (one-way Analysis of Variance) using the General Linear Models procedure of SAS (version 6.11, SAS Institute Inc., Cary, NC). When significant diet effects were detected ($P < 0.05$), the least significant difference was used to compare treatment means. Simple correlation coefficients between aberrant crypt foci, PCNA and Ki-67 labeling indices and the relative PCNA levels and were calculated and tested for significance at $P < 0.05$ using the correlation procedure of SAS. Rats from the No AOM treatment were not used in the correlation analysis because they did not develop ACF.

D. RESULTS

Isoflavone Composition of Diets

The full fat soy flake-based diet (least processed) and defatted soy flour-based diet (obtained by hexane extraction of oil from full fat soy flakes) both contained approximately 490 mg/kg genistein derivatives (Table 15). These genistein derivatives occurred primarily as glycosides in these diets. Both of these diets also contained considerable concentrations of daidzein derivatives, with the soy flake-containing diet having 422 mg/kg daidzein derivatives and the soy flour-containing diet having 334 mg/kg daidzein derivatives. Both of these diets also contained approximately 50 mg/kg glycitein derivatives. The soy concentrate-based diet, as well as the soy concentrate plus calcium diet, contained negligible amounts of isoflavones (Table 15). The soy concentrate plus genistein diet contain 151 mg/kg of genistein, most of which was directly added to the diet during its formulation.

Aberrant Crypt Foci

The effects of dietary treatment on preneoplastic lesions are shown in Table 16, which reports the total number of ACF, the number of ACF containing 4 or more aberrant crypts, and the total number of aberrant crypts per rat. These results show that rats consuming diets containing 5 g/kg calcium or 150 mg/kg added genistein had the fewest colon ACF (70 and 77 ACF, respectively). Rats consuming soy flour and full-fat soy flakes had significantly fewer ACF than rats consuming soy concentrate (87 and 97 versus 132 ACF), but both groups had significantly greater numbers of ACF than rats consuming soy concentrate plus 5 g/kg calcium. Rats consuming the soy concentrate diet

had the greatest numbers of ACF (132 ACF/rat). No ACF were identified in colons from the negative control rats that were injected with saline (data not shown).

Rats consuming the soy concentrate-based diet had the greatest ($P < 0.05$) numbers of large ACF (those having 4 or more aberrant crypts) and the greatest number of total aberrant crypts (676 aberrant crypts/rat). Rats consuming soy concentrate plus either 150 mg/kg genistein or 5 g/kg calcium had the smallest numbers of large ACF (41 and 48 large ACF/rat, respectively). Rats consuming soy flakes, soy concentrate plus genistein, or soy concentrate plus calcium had the smallest total numbers of aberrant crypts (397, 336 and 335 aberrant crypts/rat, respectively. Rats consuming soy flakes had intermediate numbers of large ACF (65/rat) and total aberrant crypts (475/rat).

Proliferation indices with IHC

Crypt Height

Data on crypt heights calculated during PCNA and Ki-67 immunohistochemistry are presented in Tables 17 and 18, respectively. Among all treatment groups, rats consuming soy concentrate and soy concentrate plus calcium had the greatest ($P < 0.05$) crypt heights, with both groups averaging over 70 cells per hemicrypt. Rats consuming soy flour and soy concentrate plus genistein had the shortest crypt heights ($P < 0.05$), with both groups averaging approximately 61 cells per hemicrypt.

Total Crypt Labeling Index (LI)

Data on PCNA labeling index in colonic epithelium are presented in Table 17. Animals consuming the soy flakes-based diet had the lowest ($P < 0.05$) total PCNA

labeling index (18%) among all groups. The greatest PCNA total LI values were observed in rats consuming soy concentrate (33.6%), followed by rats consuming soy concentrate without having AOM injections (No AOM group, LI = 27.5%). Animals consuming the soy concentrate plus genistein, soy concentrate plus calcium, and soy flour diets had statistically comparable total labeling indices (22.2, 21.5 and 20.3%, respectively), and these LI values were significantly lower than that observed in rats consuming the soy concentrate diet.

Ki-67 total LI values are presented in Table 18. Among all treatment groups, rats consuming soy concentrate had the greatest ($P < 0.05$) Ki-67 labeling index (13.9%). Rats consuming all other diets as well as rats who were not injected with AOM had statistically similar Ki-67 total labeling indices, with values ranging from 6.8% for rats consuming soy flakes to 8.6% for rats consuming soy concentrate plus calcium.

Proliferation Zone

PCNA proliferation zone data are presented in Table 17. The greatest ($P < 0.05$) PCNA PZ was observed in rats consuming the soy concentrate-based diet (50.9%) and the lowest PCNA PZ occurred in rats consuming soy flakes (34.2%) or soy flour (34.8%). Addition of genistein or calcium to the soy concentrate diet reduced PCNA PZ significantly when compared to rats consuming soy concentrate alone. Rats consuming soy concentrate without AOM injection (no AOM) had greater ($P < 0.05$) PCNA PZ (44.6%) than all treatments except for soy concentrate (with AOM injection).

Ki-67 PZ (Table 18) was greatest ($P < 0.05$) in rats consuming soy concentrate (40.6%) and lowest in rats consuming soy flakes (23.3%) or soy flour (24.8%). Rats

consuming soy concentrate plus genistein (29.2%), soy concentrate plus calcium (29.7%) or soy concentrate without AOM injection (29.8%) all had intermediate Ki-67 PZ values.

Compartmental Labeling Indices

Data on compartmental labeling indices (basal, middle and top LI values with the crypts) for PCNA and Ki-67 are presented in Tables 17 and 18, respectively. PCNA labeling in the basal third of the crypt was greatest ($P < 0.05$) in rats consuming soy concentrate either with (60.1%) or without (58.4%) AOM injection. Rats consuming soy flakes had the lowest ($P < 0.05$) basal PCNA LI (46.3%). PCNA LI in the middle third of the crypt was significantly greater in rats consuming soy concentrate (40.7%) than for all other treatments. Rats consuming soy flakes (7.8%), soy flour (9.4%) or soy concentrate plus genistein had the lowest PCNA labeling indices in the middle third of the crypts.

Ki-67 LI in the basal third of the crypt was greatest in rats consuming soy concentrate (29.0%), lowest for rats consuming soy flakes (19.2%) or soy concentrate without AOM (19.8%), and was intermediate for the other treatments. Rats consuming soy concentrate had greater ($P < 0.05$) Ki-67 LI in the middle third of the crypts (12.8%) than all other treatment groups except for rats consuming soy concentrate plus calcium (7.4%). PCNA and Ki-67 labeling in the top third of colonic crypts was minimal and did not differ among the treatment groups.

Quantified PCNA Levels

The results of slot-blot quantification of PCNA protein levels in colonic mucosa are presented in Table 19. Rats fed soy flakes or defatted soy flour had the lowest ($P <$

0.05) relative PCNA levels when compared to all other groups. Rats consuming soy concentrate had significantly greater PCNA expression than was observed in all other treatment groups. Feeding soy concentrate without AOM injection (no AOM group) resulted in greater ($P < 0.05$) PCNA expression than was observed in all AOM-injected groups except for rats consuming soy concentrate. Animals on soy concentrate plus genistein and on soy concentrate plus calcium had similar relative PCNA levels (666 and 659), although these values were significantly greater than was observed in rats consuming soy flakes or defatted soy flour.

Correlations among Biomarkers

Table 20 shows the correlation coefficients for ACF, quantified PCNA levels, PCNA total crypt LI and PZ, and Ki-67 total crypt LI and PZ. These correlations only included rats that were injected with AOM, so the data for the sham-injected control rats were excluded from this analysis. All correlations among these parameters were statistically significant. PCNA total crypt LI and PZ were highly correlated (r=0.92). Quantified PCNA protein levels also were highly correlated with total crypt PCNA LI (r=0.82) and PZ (r=0.70). Ki-67 total crypt PZ was also highly correlated with quantified PCNA (r=0.74), total crypt PCNA LI (r=0.68), total crypt PCNA PZ (r=0.64) and total crypt Ki-67 PZ (r=0.73).

E. DISCUSSION

The major goal of this research was to determine whether diets containing soy products (with varying levels of isoflavones and other phytochemicals) would inhibit the

early stages of AOM-induced colon cancer in F344 rats. Processing methods, such as alcohol extraction to produce soy protein concentrate, dramatically influence the phytochemical content in the resulting soy products. Four diets were designed to study the effect soy-based diets differing in isoflavone and other phytochemical contents on their potential impact on colonic epithelial cell proliferation and aberrant crypt foci formation. We also tested the effect of calcium concentration in the diet by including a treatment that contained 5 g/kg calcium, as compared with the low calcium level (1 g/kg) in the other treatment diets.

Colon carcinogenesis is characterized by increased colonocyte proliferation and decreased differentiation and apoptosis. The level of expression of certain proliferation or differentiation markers (such as PCNA and Ki-67) as well as the location of their expression within colonic crypts becomes important in assessing the status of preneoplasia (formation of aberrant crypt foci) and neoplasia of the colon (Yamashita et al., 1994). Detection of PCNA in the nucleolus of a colonocyte indicates the cell's capacity to go through another round of cell division or, in other words, a lack of terminal differentiation. As demonstrated in other studies (Deschner, 1988; Mills et al., 1995; Akedo et al., 2001), an upward shift in the proliferation zone in conjunction with overall increases in total cell proliferation in the colonic crypt is a useful intermediate biomarker of increased CC risk.

The cell concentration of PCNA is directly correlated with the proliferative state of the cell, increasing during G_1, peaking at the G_1/S-phase interface, decreasing during G_2 and reaching low levels in M- phase and interphase (Prelich et al., 1987). The presence of PCNA in G_0 -phase cells would indicate that these cells maintain the capacity

to go through another round of cell division. Terminally differentiated cells do not express PCNA. Thus, the absence of PCNA indicates that a cell has undergone terminal differentiation (Bromely et al., 1996). Hence, loss of PCNA expression appears to be potentially useful as a differentiation marker of colonic epithelial cells. Furthermore, detection and quantitation of PCNA content can represent proliferative status in the colonic crypts. For example, a dietary treatment that results in an upward shift in the PCNA PZ within colonic crypts suggests that terminal cell differentiation is delayed by this treatment. Ki-67 is a cell proliferation marker that is primarily expressed during the S-phase of cell cycle and absent in G_0 while PCNA accumulates as the cells progress through G_1, S, and M phases of the cell cycle and returns to low levels during G_0. While Ki-67 labeling provides an estimate of total cell proliferation in tissues (Bruno and Darzynkiewicz, 1992; Zacchetti et al., 2003) unlike PCNA, it does not necessarily reflect a cell's capacity to undergo another round of cell division (Prelich et al., 1987; Toschi and Bravo, 1988; Zacchetti et al., 2003).

In our study the rats that were fed the soy concentrate diet, after initiation of colon cancer by AOM, had the largest number of total ACF, large ACF and total aberrant crypts ($P<0.05$) among all treatment groups. The rats consuming soy concentrate also had the greatest PCNA and Ki-67 total LI and PZ values among all groups. Conversely, rats fed soy flakes, soy flour or soy concentrate plus 150 mg/kg genistein all had significantly lower Ki-67 and PCNA LI and PZ values than rats consuming soy concentrate (Tables 17 and 18). Likewise, rats fed the soy flakes, defatted soy flour and soy concentrate plus genistein all had significantly lower numbers of ACF, large ACF and total aberrant crypts when compared to the soy concentrate-fed rats. It is also

noteable that addition of 150 mg/kg genistein to the soy concentrate diet was sufficient to reduce ACF development in rats to an even greater extent than was observed by feeding soy flakes or soy flour.

Extraction of soy flakes and soy flour with aqueous ethanol to produce soy concentrate results in loss of phytochemicals such as isoflavones (genistein and daidzein), Bowman-Birk protease inhibitor, and saponins (Wang and Murphy, 1996; U.S. Department of Agriculture, 1999). As shown in Table 15, the soy concentrate diet contained minimal concentrations of isoflavones when compared to either the soy flake or defatted soy flour diet. This may account for the greater promotion of ACF and greater PCNA and Ki-67 LI and PZ values observed in the soy concentrate-fed group. The presence of high PCNA mitotic indices and upward shift of PZ observed in rats fed the soy concentrate indicates that rats consuming this diet had a substantial delay in terminal differentiation of colonic epithelial cells. It also was interesting to observe that extracting the oil- and fat-soluble components (such as sterols that has been postulated to protect against CC, (Awad et al., 1997) from soy flakes to produce soy flour did not alter potential of soy flour to reduce ACF or lower PCNA and Ki-67 LI and PZ indices.

Studies in laboratory animals have shown that feeding a soy-rich diet inhibits chemically induced carcinogenesis (Pereira et al., 1994; Koratkar and Rao, 1997; Kennedy et al., 2002). Hakkak et al. (2001) fed male Sprague Dawley rats AIN-93G diets formulated with either soy protein isolate or casein as the sole protein source for their entire life. They observed that rats fed a diet containing SPI had reduced AOM-induced colonic tumor incidence when compared with rats fed a casein-containing diet.

Rats fed the casein diet had a 50% incidence of colon tumors compared with 12% on soy protein based diet.

Genistein, a major isoflavone in soy, has been demonstrated to have anti-cancer effects in many cancer cell culture studies (Yanagihara et al., 1993). Little research has been reported that confirms a protective effect of isoflavones against carcinogenesis in animal models. Pereira et al. (1994) found that 150 mg/kg of dietary genistein inhibited the formation of ACF by 34% in the colons of rats treated with azoxymethane when compared to rats consuming the standard AIN 93-G diet.

In our study, adding the isoflavone genistein to the soy concentrate diet caused significant decreases in the number of ACF, the number of large ACF, and the total number of aberrant crypts when compared to the soy concentrate-fed rats (Table 16). Both the soy flour-based diet and the full fat soy flake-base diets contained 490 mg/kg genistein derivatives (primarily as glycosides), but the soy flake diet was less effective, and the soy flour diet was equally effective, in inhibiting the formation of total ACF compared to when 150 mg/kg genistein (as the aglycone) was added to the soy concentrate diet. The genistein-containing diet was more effective than either soy flakes or soy flour in inhibiting the formation of large ACF. These ACF results suggest that the aglycone genistein was more effective in preventing early CC promotion than genistein when present predominantly as glycosides. Feeding pure genistein compared with genistin at equimolar doses would unequivocally answer the question of whether genistein glycosides have less efficacy in inhibiting early CC promotion when compared to the aglycone form. Although genistein has been shown to inhibit the appearance of precancerous lesions in the colon of rats, we do not know if this would translate into a

reduction in colon tumors in animals or humans. A study conducted by Rao et al. (1997) demonstrated that adding genistein to the diet (250 mg/kg) actually increased AOM-induced colon cancer in rats.

The LI and PZ (both PCNA and Ki-67, Tables 17 and 18) of soy flour, calcium and genistein fed rats were not statistically different from each other and were lower ($P < 0.05$) than the LI and PZ values observed in rats fed soy concentrate. Adding 150 mg/kg of the isoflavone genistein (as the aglycone) to the soy concentrate diets caused a significant decrease in the proliferation indices when compared to feeding soy concentrate alone. In fact, the PCNA LI and PZ observed in rats fed soy flour, calcium and genistein (all of which were injected with AOM) all were lower than was observed even in the sham-injected (no AOM) rats, which were fed the soy concentrate-based diet. The Ki-67 LI and PZ were similar for all 4 of these treatment groups.

The standard AIN-93G diet has adequate calcium content (5 g/kg) to allow maximum bone and teeth mineralization in rapidly growing rodents. However, dietary calcium content can have anticancer action in the colon. Others have shown that calcium supplementation to the already high-calcium content of AIN-93G diets decreases AOM-induced ACF and tumors in rats (Beatty et al., 1993; Li et al., 1998; Wargovich et al., 2000; Pierre et al., 2003). Some clinical trials show that supplemental calcium (1.25g) may reduce the risk of CC by decreasing cell proliferation in the upper 40% of colon crypts in people at immediate or high risk for developing CC (Lipkin and Newmark, 1985; Rozen et al., 1989; Wargovich et al., 1995a). However, 3 other studies examining calcium and CC risk did not find a reduction in colonic mucosa proliferation rates or a downward shift in the proliferative compartment with calcium supplementation (1.2 –

1.5g, Stern et al., 1990; Baron et al., 1995; Cats et al., 1995). Even though the human data are less supportive of the hypotheses that high-calcium diets have a protective effect against CC development, we intentionally kept the dietary calcium content low (all diets contained 1 g/kg calcium except the 'calcium' diet which was the soy concentrate diet with 5 g/kg calcium) so that the anticancer activity of other dietary ingredients (such as the soy phytochemicals) would not be masked by dietary calcium amounts used in the standard AIN-93G diet.

Feeding 5 g/kg calcium to rats lowered ($P < 0.05$) the proliferative indices (both PCNA and Ki-67 LI & PZ) when compared to the soy concentrate-fed rats (whose diet contained 1 g/kg calcium). The soy concentrate plus calcium diet also had significantly fewer total ACF, large ACF and total aberrant crypts when compared to feeding the soy concentrate diet (which contained 1 g/kg calcium). This effect of adding calcium to the soy concentrate diet on ACF development and proliferation indices was similar to the effect of adding genistein to the soy concentrate diet. When Pereira et al. (1994) added 150 mg/kg genistein to AIN-93G diets containing 5 g/kg calcium, the number of ACF was decreased by 34% compared to rats consuming standard AIN-93G diets. In this study, the soy concentrate plus genistein diet contained 150 mg/kg genistein and 1 g/kg calcium and we observed a 42% reduction in the number of ACF with this diet compared to rats consuming soy concentrate (with 1 g/kg calcium). Thus, the response of AOM-induced ACF formation to dietary genistein observed in our study was similar to that observed by Pereira et al. (1994), although these two studies utilized control diets differing in protein source (soy concentrate versus casein) and calcium level (1 g/kg in our study and 5 g/kg in the study by Pereira et al., 1994). Given our treatment protocol,

we were unable to determine if the effects of dietary genistein and calcium might be additive. Testing the potential additivity of these effects should be the focus of a future study. We also are unable to speculate whether diets based on soy flakes or soy flour would reduce ACF formation when the diet contains a higher calcium level (i.e. 5 g/kg as in the standard AIN-93G diet).

The quantity of PCNA protein in colonic mucosa represents a potentially useful marker for the study of mucosal proliferative activity and therefore, colon cancer risk. Using slot-blot methodology, we quantified the PCNA protein levels in the colonic mucosa of rats in this study. The results obtained from this method indicate that relative amounts of PCNA quantified by slot-blotting reflect the dietary induced differences we observed by PCNA immunohistochemistry (Tables 17 and 19) results. Rats fed soy flour and soy flakes had the lowest ($P < 0.05$) levels of quantified PCNA (slot-blot method) among all treatments, whereas rats fed soy concentrate had the greatest ($P < 0.05$) quantified PCNA levels. Furthermore, quantified PCNA was highly correlated with PCNA and Ki-67 LI and PZ values (Table 20), demonstrating that slot-blot quantification of PCNA represents another potentially useful method to evaluate the efficacy of diet to influence colon cancer risk.

In overall conclusion, this study indicates that diets based on soy flour and soy flakes, as compared with diets based on soy concentrate, are protective against AOM-induced ACF formation. Although the particular phytochemicals present in soy flour and soy flakes that are responsible for this protective effect were not identified in this study, we demonstrated that we could achieve similar levels of protection against ACF formation by supplementing a soy concentrate-based diet with 150 mg/kg genistein.

Supplementation of the soy concentrate-based diet with calcium to a level of 5 g/kg calcium in the diet was able to exert a level of protection against ACF formation that was comparable to that observed when feeding soy flour, soy flakes and soy concentrate plus genistein. Reductions in ACF formation by diet were associated strongly with reductions in whole crypt LI and PZ for both PCNA and Ki-67, and also were correlated with significant reductions in PCNA protein expression in colonic mucosa.

Table 14. Composition of diets[1]

Ingredients	Treatment Groups				
	Soy flakes	Soy flour	Soy concentrate	Soy concentrate + additional	
				GEN[2]	CAL[3]
			% by wt		
Full Fat Soy Flakes	46.07	-	-	-	-
Soy Flour	-	35.79	-	-	-
Soy Concentrate	-	-	28.45	28.45	28.45
Genistein	-	-	-	0.015	-
Calcium Carbonate	-	-	-	-	1.00
Soy Oil	4.95	14.72	14.78	14.78	14.78
Corn Starch	2.85	3.36	10.64	10.63	9.64

[1] A basal mixture comprised 46.13% of the diet and contained sucrose (40% of diet), mineral mix (4.2% of diet providing 0.1% Ca by weight in the diet), vitamin mix (1.13% of diet), methionine (0.23% of diet), L-cystine (0.34% of diet), choline bitartrate (0.23% of diet) and butylated hydroxytoluene (0.003% of diet).

[2] GEN – Soy concentrate plus additional genistein (150mg/Kg diet)

[3] CAL – Soy concentrate plus calcium to equal the standard AIN-93G mineral mix (5g Ca/Kg diet)

Table 15. Isoflavone concentrations in rat diets

Isoflavone	Diets: Soy flakes	Soy flour	Soy concentrate and SC plus calcium	Genistein
	mg of aglycone/ kg of diet			
Malonyl-genistin†	212	342	8	8
Genistin†	235	142	8	8
Genistein	45	10	1	151
Total geinstein derivatives	492	494	17	167
Malonyl-daidzin†	126	196	7	7
Daidzin†	182	92	7	7
Daidzein	114	46	5	5
Total daidzein derivative	422	334	19	19
Glycitin†	48	45	3	3
Glycitein	4	ND‡	1	1
Total glycitein derivatives	52	45	4	4

† Expressed as mg of the aglycone, not the glycoside weight
‡ ND – none detected

Table 16. Effect of diet on preneoplastic lesions in the colon of rats treated with AOM

Diet[†]	Preneoplastic lesions[‡]*		
	ACF	ACF ≥ 4 AC	Total AC
SFK	$97^{b} \pm 5$	$65^{b} \pm 3$	$475^{b} \pm 24$
SFL	$87^{bc} \pm 5$	$54^{c} \pm 3$	$397^{c} \pm 24$
SC	$132^{a} \pm 5$	$91^{a} \pm 3$	$676^{a} \pm 24$
SC+GEN	$77^{cd} \pm 5$	$41^{d} \pm 3$	$336^{c} \pm 24$
SC+CAL	$70^{d} \pm 5$	$48^{cd} \pm 3$	$335^{c} \pm 24$

[†] SFK – soy flakes; SFL – soy flour; SC – soy concentrate; SC+GEN – soy concentrate plus genistein; SC+CAL – soy concentrate plus calcium.

[‡] ACF – Foci with one or more aberrant crypts/rat; ACF ≥ 4 AC – ACF with 4 or more aberrant crypts/rat; total AC – the total number of aberrant crypts/rat.

* Values are presented as means and ± SEM (n = 15 rats/treatment). Means in the same column not sharing a common superscript are significantly different ($P < 0.05$).

Table 17. Effect of diet on colonic crypt height and epithelial PCNA proliferation indices in rats treated with AOM

Diet‡	PCNA Indices†		
	CH§	LI	PZ
No AOM	$66.6^{b} \pm 1.2$	$0.275^{b} \pm 0.008$	$0.446^{b} \pm 0.015$
SFK	$65.6^{bc} \pm 1.1$	$0.180^{d} \pm 0.008$	$0.342^{d} \pm 0.014$
SFL	$60.8^{d} \pm 1.2$	$0.203^{c} \pm 0.008$	$0.348^{cd} \pm 0.015$
SC	$69.7^{ab} \pm 1.2$	$0.336^{a} \pm 0.008$	$0.509^{a} \pm 0.015$
SC+GEN	$61.8^{d} \pm 1.2$	$0.222^{c} \pm 0.008$	$0.389^{c} \pm 0.015$
SC+CAL	$72.4^{a} \pm 1.3$	$0.215^{c} \pm 0.008$	$0.388^{c} \pm 0.016$

Diet‡	PCNA Indices†		
	Basal LI	Mid LI	Top LI
No AOM	$0.584^{ab} \pm 0.014$	$0.233^{b} \pm 0.021$	0.007 ± 0.002
SFK	$0.463^{d} \pm 0.014$	$0.078^{d} \pm 0.020$	0.000 ± 0.002
SFL	$0.530^{c} \pm 0.014$	$0.094^{cd} \pm 0.021$	0.000 ± 0.002
SC	$0.601^{a} \pm 0.014$	$0.407^{a} \pm 0.021$	0.000 ± 0.002
SC+GEN	$0.545^{bc} \pm 0.014$	$0.125^{cd} \pm 0.021$	0.000 ± 0.002
SC+CAL	$0.553^{bc} \pm 0.015$	$0.150^{c} \pm 0.022$	0.000 ± 0.002

† Values are presented as means and ± SEM (n = 10 rats/treatment except n = 9 for SC+CAL and n = 11 for SFK). Means in the same column not sharing the same superscripts are significantly different ($P < 0.05$).

‡ No AOM – vehicle treated; SFK – soy flakes; SFL – soy flour; SC – soy concentrate; SC+GEN – soy concentrate plus genistein; SC+CAL – soy concentrate plus calcium.

§ CH – Crypt Height, total number of cells in the hemicrypt from the base to the mouth of the crypt;
LI – Total Crypt Labeling Index, obtained by dividing the number of labeled cells in a hemicrypt by crypt height.
PZ – Proliferation Zone, obtained by dividing the position of the highest labeled cell from the hemicrypt base by the crypt height
Basal LI– Labeling Index in Basal one-third compartment of the crypt
Mid LI – Labeling Index in Middle one-third compartment of the crypt
Top LI – Labeling Index in Top one-third compartment of the crypt

Table 18. Effect of diet on colonic crypt height and epithelial Ki-67 proliferation indices in rats treated with AOM

Diet‡	Ki-67 Indices†		
	CH§	LI	PZ
No AOM	$65.9^{b} \pm 1.1$	$0.076^{b} \pm 0.017$	$0.298^{b} \pm 0.020$
SFK	$66.4^{b} \pm 1.1$	$0.068^{b} \pm 0.016$	$0.233^{c} \pm 0.191$
SFL	$61.1^{c} \pm 1.1$	$0.073^{b} \pm 0.017$	$0.248^{bc} \pm 0.020$
SC	$70.4^{a} \pm 1.1$	$0.139^{a} \pm 0.017$	$0.406^{a} \pm 0.020$
SC+GEN	$61.4^{c} \pm 1.1$	$0.083^{b} \pm 0.017$	$0.292^{b} \pm 0.020$
SC+CAL	$72.4^{a} \pm 1.2$	$0.086^{b} \pm 0.018$	$0.297^{b} \pm 0.021$

Diet‡	Ki-67 Indices†		
	Basal LI	Mid LI	Top LI
No AOM	$0.198^{b} \pm 0.029$	$0.030^{b} \pm 0.023$	0.000 ± 0.001
SFK	$0.192^{b} \pm 0.028$	$0.013^{b} \pm 0.022$	0.001 ± 0.001
SFL	$0.208^{ab} \pm 0.029$	$0.021^{b} \pm 0.023$	0.000 ± 0.001
SC	$0.290^{a} \pm 0.029$	$0.128^{a} \pm 0.023$	0.000 ± 0.001
SC+GEN	$0.226^{ab} \pm 0.029$	$0.029^{b} \pm 0.023$	0.000 ± 0.001
SC+CAL	$0.228^{ab} \pm 0.030$	$0.074^{ab} \pm 0.025$	0.000 ± 0.001

† Values are presented as means and ± SEM (n = 10 rats/treatment except n = 9 for SC+CAL and n = 11 for SFK). Means in the same column not sharing the same superscripts are significantly different ($P < 0.05$).

‡ No AOM – vehicle treated; SFK – soy flakes; SFL – soy flour; SC – soy concentrate; SC+GEN – soy concentrate plus genistein; SC+CAL – soy concentrate plus calcium.

§ CH – Crypt Height, total number of cells in the hemicrypt from the base to the mouth of the crypt;

LI – Total Crypt Labeling Index, obtained by dividing the number of labeled cells in a hemicrypt by crypt height.

PZ – Proliferation Zone, obtained by dividing the position of the highest labeled cell from the hemicrypt base by the crypt height

Basal LI– Labeling Index in Basal one-third compartment of the crypt

Mid LI – Labeling Index in Middle one-third compartment of the crypt

Top LI – Labeling Index in Top one-third compartment of the crypt

Table 19. Effect of diet on quantified colonic PCNA levels in rats treated with AOM

Diet[†]	PCNA[‡]
No AOM	736^{b} ±18
SFK	535^{d} ±17
SFL	536^{d} ±18
SC	1014^{a} ±18
SC+GEN	666^{c} ±18
SC+CAL	659^{c} ±19

[†] No_AOM – vehicle treated; SFK – soy flakes; SFL – soy flour; SC – soy concentrate; SC+GEN – soy concentrate plus genistein; SC+CAL – soy concentrate plus calcium.

[‡] Quantified PCNA units are relative. Values are presented as means and ± SEM (n = 10 rats/treatment except n = 9 for SC+CAL and n = 11 for SFK). Means in the same column not sharing the same superscripts are significantly different ($P < 0.05$).

Table 20. Correlation coefficients for total ACF, quantified PCNA levels, PCNA LI and PZ and Ki-67 LI and PZ in colonic mucosa of rats injected with AOM

Parameter†	PCNA SB	PCNA LI	PCNA PZ	Ki-67 LI	Ki-67 PZ
Total ACF	0.51	0.56	0.52	0.34	0.43
P value	0.0002	<0.0001	0.0001	0.0143	0.0017
PCNA SB	-	0.82	0.70	0.53	0.74
P value		<0.0001	<0.0001	<0.0001	<0.0001
PCNA LI	-	-	0.92	0.38	0.68
P value			<0.0001	0.0064	<0.0001
PCNA PZ	-	-	-	0.34	0.64
P value				0.0165	<0.0001
Ki-67 LI	-	-	-	-	0.73
P value					<0.0001

† Total ACF – total aberrant crypt foci; PCNA SB – PCNA quantified by slot blotting; LI – Labeling Index; PZ – Proliferative Zone. Total N = 50; n = 10 rats/treatment except n = 9 for SC+CAL and n = 11 for SFK. Rats from the No AOM treatment were not used in this analysis.

CHAPTER VI. SUMMARY AND CONCLUSIONS

Colon cancer (CC) incidence as an end point in chemoprevention trials is impractical due to the length of time required for CC to develop as well as the large number of subjects required to test the efficacy of various interventions. Thus, there is a need to identify biomarkers that will reliably predict CC risk and benefits of intervention trials. The overall goal of this research was to determine the colon cancer chemo-preventive potential of soy-containing foods in humans at risk for CC and in animals initiated with a colon carcinogen using intermediate biomarkers rather than appearance of polyps or cancer as the endpoint. Increased proliferation (as measured by tritiated thymidine and BrdU labeling) and delayed terminal differentiation in colonic epithelial cells are commonly found in individuals with increased risk for CC and in rodents treated with carcinogens that induce CC.

The first objective of the first study was to determine the correlation between CC risk and indices of mucosal proliferation (LI and PZ as assessed by IHC detection of PCNA and Ki-67). Given the laborious nature of IHC techniques used to measure proliferation markers, there is a need to develop a more facile index of colonic epithelial cell proliferation. A simpler method would be highly desirable in dietary intervention and large scale clinical studies that evaluate the risk for CC. For this reason, the second objective of this research was to use an immunoblotting method to determine relative levels of PCNA protein in colonic epithelial cells and determine if PCNA protein correlates with proliferation indices based on IHC techniques. The first study demonstrated that the proliferative compartment (measured by PCNA and Ki-67 PZ), PCNA LI, and relative PCNA protein levels were positively correlated with increasing

age and CC risk and were therefore considered useful intermediate biomarkers to assess CC risk. Ki-67 LI was judged to be not useful for assessing CC risk status.

The objective of the second study was to utilize proliferative indices (PCNA and Ki-67 labeling and quantitative PCNA protein expression) as intermediate biomarkers to test for reduction of CC risk in humans after soy consumption for one year. A double-blind, prospective study was conducted to determine if consumption of one of two supplements containing soy protein isolate with isoflavones or a supplement containing calcium caseinate for one year would influence colon epithelial cell proliferation. The two soy supplements differed only in their calcium contents. There were statistically significant reductions in both PCNA LI and PCNA PZ during the course of the experiment in subjects that consumed soy supplements but not in subjects who consumed casein. There were no significant treatment effects on total Ki-67 LI or Ki-67 PZ. The relative expression of PCNA protein in colonic mucosa was significantly reduced during the course of the experiment in subjects who consumed either of the soy supplements, but was not influenced by consumption of the casein supplement. Since the presence of PCNA indicates the capacity of cells to undergo cell division, the decrease in PCNA LI and PZ observed in subjects consuming the soy supplements denotes a lowering of the cell proliferation capacity and infers a greater degree of terminal cell differentiation of colonic crypts in those subjects. This study indicated that consumption of supplements containing soy protein isolate caused changes in colon epithelial cell proliferation patterns that are consistent with a reduced risk for developing colon cancer.

The objective of the final study was to investigate the CC chemopreventive potential of different soy products in carcinogen-treated rats as reflected by changes in

ACF and colon epithelial cell proliferation. Rats consuming soy concentrate had the greatest numbers of total ACF, large ACF and total aberrant crypts among all treatment groups. Supplementation of the soy concentrate diet with either genistein or calcium resulted in the lowest numbers of these endpoints. All biomarkers of cell proliferation tested in this study (PCNA and Ki-67 LI and PZ, quantified PCNA protein) were positively correlated with each other and with numbers of ACF. The data from this study indicates that one or more aqueous-alcohol soluble, but not fat soluble, phytochemicals present in soy is responsible for the reduction in ACF and reduced colonic epithelial cell proliferation observed in AOM-treated rats. Furthermore, the protective effects of soy flakes and soy flour against AOM-induced ACF development were replicated by addition of 150 mg/kg genistein or 5 g/kg calcium to the soy concentrate diet.

In conclusion, it was demonstrated that PCNA LI and PZ, Ki-67 PZ, and relative concentrations of PCNA protein in colonic mucosa are highly correlated and are correlated with risk of CC, which indicates that these biomarkers have utility as intermediate biomarkers in chemoprevention studies. Consumption of a soy protein isolate-based supplement for one year reduced risk of CC in humans and feeding soy flakes and soy flour reduced biomarkers of CC in a rat model of human CC.

CHAPTER VII. RECOMMENDATIONS FOR FUTURE STUDIES

The following studies should be conducted to further assess the potential efficacy of soy products and specific soy phytochemicals to reduce colon cancer risk.

A. Examination of the potential efficacy of soy protein isolate, mixed soy isoflavones, and genistin to reduce indices of colon cancer risk (PCNA LI and PZ, PCNA protein expression) in colonic mucosa of humans at high risk for colon cancer.

In this dissertation research, I demonstrated that consumption of soy protein isolate-based supplements for one year by subjects at high risk for colon cancer resulted in reduced indices of colonic epithelial cell proliferation (Chapter 4). Although the supplement treatments used in this research were similar in their contents of protein and most essential nutrients, they did differ significantly in calcium concentration. The soy protein isolate supplement containing calcium was more effective in reducing indices of cell proliferation than was the soy protein isolate supplement without additional calcium. Thus, the effects of soy could not be completely separated from potential effects of calcium in this research. This experiment should be repeated with a similar group of high-risk individuals who consume supplements designed to directly assess the effects of soy protein isolate, isoflavones, and genistin on indices of colonic epithelial cell proliferation. The specific effects of genistin are particularly interesting because this research also demonstrated that 150 mg of aglycone genistein/kg diet added to a soy concentrate-based diet significantly reduced indices of colonic epithelial cell proliferation in AOM-treated rats (Chapter 5). The supplement treatments (40 g protein/day) would

consist of 1) soy protein isolate, 2) soy protein concentrate (prepared by alcohol extraction and thus containing very low concentrations of isoflavones), 3) soy protein concentrate plus mixed isoflavones added at concentrations to mimic those in soy protein isolate, and 4) soy protein concentrate plus genistin at concentrations equal to those in soy protein isolate. Subjects would consume these supplements in addition to their regular diets for one year. Colonic mucosal biopsies would be obtained before initiation of the experiment and after one year of supplementation. Biopsies would be used to assess colonic epithelial cell proliferation, differentiation and apoptosis.

B. Validation of quantitative PCNA protein expression in human colonic mucosa as a reliable preneoplastic biomarker for elevated colon cancer risk.

The research reported in Chapter 3 demonstrated that quantified PCNA protein concentrations in colonic mucosal biopsies were significantly increased in persons of older age (having no history of colonic disease) and in persons who had a history of adenomatous polyps, familial adenomatous polyposis, and colon cancer. A particularly striking observation in this research was the finding that humans who had a history of adenomatous polyps, regardless of age, had colonic epithelial cell proliferation patterns comparable to the oldest subjects having no history of colonic disease. This observation confirms that colonic epithelial cell proliferation in normal-appearing colonic mucosa is disregulated in persons who develop adenomatous polyps and more advanced colonic neoplasia. This research also demonstrated that quantified PCNA protein concentrations in colonic mucosa were highly correlated with established IHC markers of colon cancer risk (PCNA LI and PZ). This research should be extended to assess the potential of

quantitative PCNA protein expression to predict colon cancer risk in a prospective human clinical study. Although the cost and logistics of conducting such a study solely for the purpose of validating PCNA protein expression as a biomarker of neoplastic potential, is unwarranted, the possibility of joining this research objective to an ongoing prospective study of human colon cancer development should be pursued.

C. Determine if elevated intermediate biomarkers of colon cancer risk translate to increased tumorigenesis in rodent models of human colon cancer.

The research reported in Chapter 5 demonstrated that intermediate biomarkers of colon cancer risk (ACF, PCNA LI and PZ, quantified PCNA protein expression) were highly correlated in an AOM-induced rat model of human colon cancer. However, the validity of ACF and other intermediate biomarkers to predict colon cancer development (adenomcarcinomas) remains in question. In order to determine the potential of intermediate biomarkers to predict colon cancer risk, a wide range colon cancer incidence and colon tumor burden must be produced by the dietary treatments. Thus, treatments used in this experiment should include 1) high-fat (15%) AIN-93G control diet, and isonitrogenous, isoenergetic diets containing the following protein and phytochemical sources, 2) full fat soy flakes, 3) defatted soy flour, 4) soy protein isolate, 5) alcohol-extracted soy concentrate, 6) diet 5 + mixed isoflavones to mimic their concentrations found in diet 2, and 7) diet 5 + genistin to mimic its concentration in diet 2. These diets would be administered to F344 rats beginning one week after a second injection of AOM (15 mg/kg body weight) and fed for a period of 40 weeks. Rats would be sacrificed after 10 weeks and 40 weeks of tumor promotion. Intermediate biomarkers of colon cancer

risk would be assessed at the 10-week time point, and colon tumor incidence, burden and histological grade would be determined at the 40-week time point. Intermediate biomarkers (PCNA LI and PZ, quantified PCNA protein expression) also would be assessed after 40 weeks of tumor promotion to assess any changes that occur in these indices during the promotion phase.

D. Determine mechanisms of action whereby soy-containing diets and soy isoflavones influence colonic epithelial cell proliferation, differentiation and apoptosis.

Although several studies have demonstrated 1) that soy-containing diets can influence colon cancer risk in animal models, and 2) that isolated soy phytochemicals (e.g. isoflavones) can influence signaling pathways and growth of cancer cells in culture, there remains a dearth of conclusive information on the mechanisms whereby soy-containing diets and isoflavones influence colon cancer risk. Studies utilizing carcinogen-induced and knockout rodent models of human colon cancer should be utilized to systematically evaluate potential mechanisms whereby soy influences colon cancer development. The initial focus of these studies should be on the activities of cell signaling pathways known to be up-regulated in colon carcinogens (map kinase pathways, Wnt/β-catenin pathway, etc.) in colonic epithelial cells obtained from rodents exposed to soy flour- or isoflavone-containing diets as compared to diets based on soy concentrate (which is not protective against colon cancer development relative to full fat soy flakes or de-fatted soy flour). These studies also should utilize gene expression

profiling coupled with real-time PCR assays to identify genes that are influenced by dietary soy.

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