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Apigenin-induced cell cycle arrest at G_2/M in human colon cancer cells

By

Chilly Sara Chung

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Nutrition

Major Professor: Diane F. Birt

Iowa State University

Ames, Iowa

2001

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Graduate College

Iowa State University

This is to certify that the Master's thesis of

Chilly Sara Chung

has met the requirements of Iowa State University

Signatures have been redacted for privacy

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To my dad and mom

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ACKNOWLEDGEMENTS

Dear Heavenly Father,

Thank You, Father, for granting me the wonderful opportunity to know You. You have led me with your guiding hands throughout my years here in the United States. From the very beginning, You have been with me, in days of happiness and sorrow. My heart is overwhelmed with gratitude towards the unconditional love You have always shown me, as You have always provided for me in many unimaginable ways. Because of Your undying love, I am able to stand here and know that You will always be there with me.

Thank You, Father, for giving me such a wonderful family. I am forever grateful to my dad, my mom and my brother for loving me and supporting me. Even though we are apart, we know that we are always together in Your love. Thank you for taking care of us throughout our years in Paraguay, as we know that it was Your will that guided us there. You never left us or neglected us, and for that I thank You. One thing I ask, that is to please continue to bless our family today, tomorrow, and until the day we meet with You.

Thank You, Father, for providing me the wonderful family I have here in Ames. Thank You for the opportunity that You gave me to know the brothers and sisters in Christ. Because of Your love for us, we are able to love and support each other through prayers as You have taught us. I especially want to thank You for bringing Yu Yang into my life. He has given me such love and support that only You could have shown him through Your divine grace. I only ask that You continue to show us how to love and support each other, and teach us how to grow with each other in Your love.

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Thank You, Father, for choosing the Birt laboratory as the place were I would dedicate many hours here in Ames. I feel so fortunate and honored to have Dr. Diane Birt as my major professor and my mentor. I am so grateful to her for the endless times she has given me guidance and support to face the many obstacles during my scholastic years at Iowa State Univerisity. Many times You have showed me Your love and friendship through the members of the Birt laboratory. For this, I thank You for letting me know them and learn to appreciate and value our friendship.

Thank You, Father, for loving me unconditionally, despite my many faults. As I look back at the years that have passed, I feel so blessed and loved by You. You were always there with me, in each and every place I have been, in each and every moment in my life. Everyday is a blessing from You, Father, and for this I am so grateful that You grant me life each morning as I wake up. Here I pray that You teach me how to follow You and show me Your way to You.

Thank You, Father, for granting me the opportunity to dedicate this part to thank You. But most importantly Father, I thank You for letting me love You.

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ABSTRACT

A diet rich in fruits and vegetables is associated with a reduced incidence of many cancers, including colon cancer. Apigenin is a dietary flavonoid that is widely distributed in fruits and vegetables. It was previously shown to induce G_2/M cell cycle arrest in three different human colon cancer cell lines that have APC mutations (SW480, HT29 and CaCo2). In this study, we assessed the importance of the APC tumor suppressor mutation in relation to the ability of apigenin to induce cell cycle arrest. The cell model system utilized for this study consisted of HT29-APC cells, which contain an inducible wild-type APC gene characterized by an increase in apoptosis in cultured HT29-APC cells treated with 100μM $ZnCl_2$. HT29-GAL cells, containing β-galactosidase plasmid, served as the control. Apigenin treatment (from 0μM to 80μM) for 48 hours resulted in a significant ($P<0.05$) reduction in the cell number concurrent with flow cytometry results showing a dose-dependent accumulation of cells in G_2/M phase in both uninduced HT29-APC and HT29-GAL cells. Terminal Deoxytransferase-Mediated Deoxyuridine NickEnd-Labeling (TUNEL) assay determined a significant ($P<0.05$) increase in the percentage of apoptotic HT29-APC cells when treated with 0μM apigenin and 100μM of $ZnCl_2$ after 120hr (induction of wild-type APC gene). Additionally, apigenin treatment (80μM) appeared to enhance the sensitivity of the HT29-APC cells to apoptosis when treated with $ZnCl_2$ to induce wild-type APC expression. On the other hand, no significant changes in the percentage of apoptotic cells were present either in the HT29-APC cells when treated with apigenin alone or in the HT29-GAL cells when treated with either apigenin and/or $ZnCl_2$. Flow cytometric analysis showed an increase ($P<0.05$) in the percentage of cells in G2/M when HT29-APC cells were

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treated with 80μM apigenin for 120 hr. This increase was not present in HT29-APC cells when treated with both 80μM apigenin and 100μM $ZnCl_2$ for 120hr. Nevertheless, the apigenin-treated HT29-GAL cells showed a significant ($p<0.05$) accumulation of cells in G_2/M regardless of the presence of $ZnCl_2$. These results suggest that APC dysfunction may be critical for apigenin to induce cell cycle arrest in human colon cancer cell lines.

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Consumption of a diet abundant in fruits and vegetables has been associated with a reduced incidence of many cancers, including colon cancer (Slattery et al. 1997). Several epidemiological studies have indicated that the higher consumption of fruits and vegetables may lower the risk of certain cancers (Williams et al. 1999). Numerous classes of compounds present in fruits and vegetables are assumed to take the role of cancer-preventive agents (Birt et al. in press, Hill 1999). Among these, one class of compounds may include the flavonoids, which are widely distributed among plants.

Apigenin[5,7,4'-trihydroxyflavone], a common dietary flavonoid, has been found in a variety of fruits and vegetables including parsley, onions, apples, tea, and chamomile. Our laboratory has previously studied the possible role of apigenin as a chemopreventive agent in colon carcinogenesis. Animal studies revealed that colonic ornithine decarboxylase (ODC) activity and aberrant crypt foci (ACF) formation in CF-1 mice were reduced with 0.1% dietary apigenin when compared with the control (Au et al. unpublished). Wang et al. (2000) has shown that apigenin induced G_2/M cell cycle arrest in three different colon cancer cell lines with truncated APC gene (SW480, HT29, and Caco2). The different effectiveness of apigenin on cell growth and cell cycle arrest in these three cell lines was suggested to be related, in part, to the different genetic background of these cells. Thus, it lead to the implication that apigenin may have a stronger effect on tumors with certain genetic mutations.

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The cause of colorectal cancer is now widely accepted to be the accumulation of mutations in specific genes controlling cell division, apoptosis and DNA repair. One of the early events involved in colorectal tumorigenesis is associated with mutations in or inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene. The APC gene is presumably involved in a broad spectrum of cellular functions as well as playing a key role in the maintanace of homeostasis in continuously proliferating tissues, and preventing uncontrolled growth and tumor formation (Kinzler and Vogelstein 1996). It was found to be a critical element in the colon cancer pathway as more than 80% of colorectal tumors have inactivating APC gene mutations (Goss and Groden 2000). In absence of functional APC, free β-catenin accumulates in the cytoplasm, leading to its translocation to the nucleus. β-catenin is a cytoplasmic protein, which has important functions in cellular adhesion and development (Morin et al. 1996). It associates with DNA-binding protein T-cell transcriptional factors (Tcf) and this β-catenin /Tcf complex may modulate the transcription of target genes such as c-myc (He et al. 1998). Activation of the signaling pathway involving the proto-oncogene c-myc will lead to the promotion of cell growth and proliferation. c-myc expression levels are usually increased in most colorectal cancers and may be linked as a direct consequence of mutations of the APC tumor suppressor gene (He et al. 1998).

Our objective was to test the hypothesis that the APC mutations in human colon cancer cell lines are critical for apigenin to induce a G_2/M cell cycle arrest and thus affect tumor cell growth, whereas the presence of wild-type APC expression would block the ability of apigenin to induce cell cycle arrest. To address this hypothesis, we used a unique cell model system, consisting of transfected HT29 (HT29-APC and HT29-GAL) human colon cancer cell lines. HT29-APC cells contain an inducible wild-type APC, which was

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expressed by an increase in the proportion of apoptotic cells following the addition of 100μM of $ZnCl_2$ (Morin et al. 1996). HT29-GAL cells contain a galactosidase plasmid, serving as a control cell line. In this study, TUNEL assay was conducted to demonstrate the induction of apoptotic cells in the HT29APC cells treated with $ZnCl_2$. In addition, cell count and flow cytometric analysis were assessed concurrently to determine the effectiveness of apigenin on cell growth and cell cycle in these cells.

Thesis Organization

This thesis contains a general introduction, which includes a literature review, followed by a manuscript suitable for publication in a scientific journal, a general conclusion and appendix. Literature cited is listed in alphabetical order according to the author's name at the end of each chapter.

Literature Review

Diet and Colon Cancer

A diet rich in fruits and vegetables has been associated with a reduced incidence of cancers, including colon cancer (Slattery et al. 1997). This association may be due to the fact that such diets tend to be lower in energy and fat or to the different components found in fruits and vegetables. Several epidemiological studies have indicated that the higher consumption of fruits and vegetables may lower the risk of certain cancers (Williams et al.

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1999). In a study by Steinmetz et al. (1994), total intake of fruits and vegetables was inversely associated with colon cancer risk in women. A similar association was observed between fruit and vegetable intake and lung cancer (Steinmetz et al. 1993, Yong et al. 1997), where a significant 20 to 60% reduction in lung cancer risk was observed for the high versus the low total fruit and vegetable consumption. Other studies conducted in different countries have also assessed the association between fruit and vegetable intake with many types of cancers (Steinmetz and Potter 1996). Because of the complexity of plant foods, numerous naturally occurring compounds found in fruits and vegetables may contribute to cancer prevention (Birt et al. in press, Hill 1999). Among these compounds, one class is the flavonoids, which are widely distributed among plants. Flavonoids are plant secondary metabolites, composed of a common structure (C6-C3-C6) with one or more hydroxyl substituents. The average daily human intake of flavonoids in the UK and in USA has been estimated to be 20mg to one gram (Hollman and Katan 1999). Recent studies have investigated the potential mechanisms by which these agents may prevent cancer, which include their ability to control cell cycle, and thus inhibiting aberrant cell proliferation (Birt et al. in press). Compelling data have shown the effect of flavonoids on cell growth and cell cycle. Flavonoids such as quercetin have been found to induce apoptosis at concentrations of 50μM or higher in several cancer cell lines (Wei et al. 1994). Furthermore, quercetin treatment was found to be effective in preventing skin carcinogenesis in the two-stage model of DMBA-initiated and TPA-promoted cancer, as well as inhibiting azoxymethanol (AOM)-induced aberrant crypt foci (ACF) in animal models (Deschner et al. 1991). Other compounds such as genistein, an isoflavonoid, was shown to induce cell cycle arrest at G_2/M in HGC-27 human gastric cancer cells at doses up to 60 μM (Matsukawa et al. 1993).

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Apigenin [5,7,4'-trihydroxyflavone], a common dietary flavonoid (see Figure 1), is widely distributed in a variety of fruits and vegetables including parsley, onions, apples, tea, and chamomile. It is relatively nontoxic and nonmutagenic when compared with other flavonoids such as quercetin (Brown and Dietrich 1979). Sato et al. (1994) demonstrated the induction of G2/M cell cycle arrest by apigenin in rat neuronal cells. Apigenin was also shown to inhibit cell growth in solid malignant tumor cells (Fotsis et al. 1997), and in leukemia cells (Lee et al. 1995). It has been studied extensively in the Birt laboratory as a potential chemopreventive agent for skin cancer, both in vivo and in vitro. Wei et al. (1990) has shown that apigenin significantly inhibited epidermal ornithine decarboxylase (ODC) activity induced by 12-0-tetradecanoylphorbol-13-acetate (TPA) in murine epidermis. Topical application of apigenin significantly reduced the number of benign papillomas and squamous cell carcinomas in Sencar mice when treated in the two-stage skin carcinogenesis model initiated by 7, 12-dimethylbenz(a)anthracene (DMBA) and promoted by 12-0-tetradecanoylphorbol-13-acetate (TPA). Based on these studies demonstrating the chemopreventive effects of apigenin *in vivo*, *in vitro* studies were conducted to investigate the molecular mechanisms of apigenin's chemopreventive avtivities. Cell culture studies by Lepley et al. (1996) demonstrated that apigenin treatment at doses up to 45μM resulted in the induction of a G_2/M cell cycle arrest in C50 murine keratinocyte cell line. The cellular concentrations detected in these apigenin-treated C50 cultured cells were similar to the concentrations revealed in the *in vivo* mouse epidermal cells treated topically with 5μM apigenin (Li et al. 1996). Because some flavonoids may induce apoptosis, the possibility of apigenin induction of apoptosis at high doses was investigated in keratinocytes (Lepley et al. 1997). However, they were unable to detect apoptosis determined by DNA fragmentation

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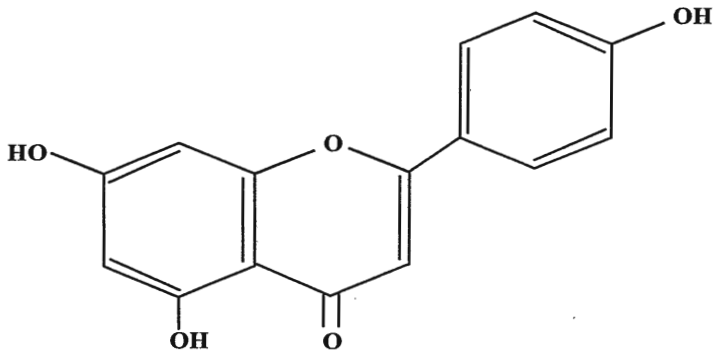
gels, TUNEL or flow cytometry. These promising results demonstrating the effectiveness of apigenin in the prevention of skin cancer have prompted further studies to investigate the efficacy of apigenin, through a dietary approach, on chemoprevention of colon carcinogenesis.



APIGENIN **(5, 7, 4'-trihydroxyflavone)**

Figure 1. Structure of Apigenin

Recent studies in the Birt laboratory have investigated the possible role of apigenin as a chemopreventive agent in colon carcinogenesis. In animal studies, 0.1% dietary apigenin reduced the colonic ornithine decarboxylase (ODC) activity and aberrant crypt foci (ACF) formation in CF-1 mice when compared with the control (Au et al. unpublished data). The measurement of the ODC activity, which is a marker for cell proliferation, along with the development of ACF, which are early preneoplastic lesions in colonic mucosa of carcinogen-treated animals, are markers that have been developed and extensively used to study colon carcinogenesis. *In vitro* studies have shown that apigenin induced G_2/M cell cycle arrest in

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three different colon cancer cell lines (SW480, HT29, and Caco2) with truncated APC gene (Wang et al. 2000). The apigenin-induced cell cycle arrest was reported to be reversible in the colon cancer cells, indicating that the effect of apigenin appeared to be cytostatic rather than cytotoxic. The mechanisms by which apigenin induces a G_2/M cell cycle arrest were also determined in colon cancer cells (Wang et al. 2000), as the association of p34 cdc2 kinase with cyclin B is required for regulation and activation of cell passage through G_2/M following a series of phosphorylation and dephosphorylation events (see Figure 2).

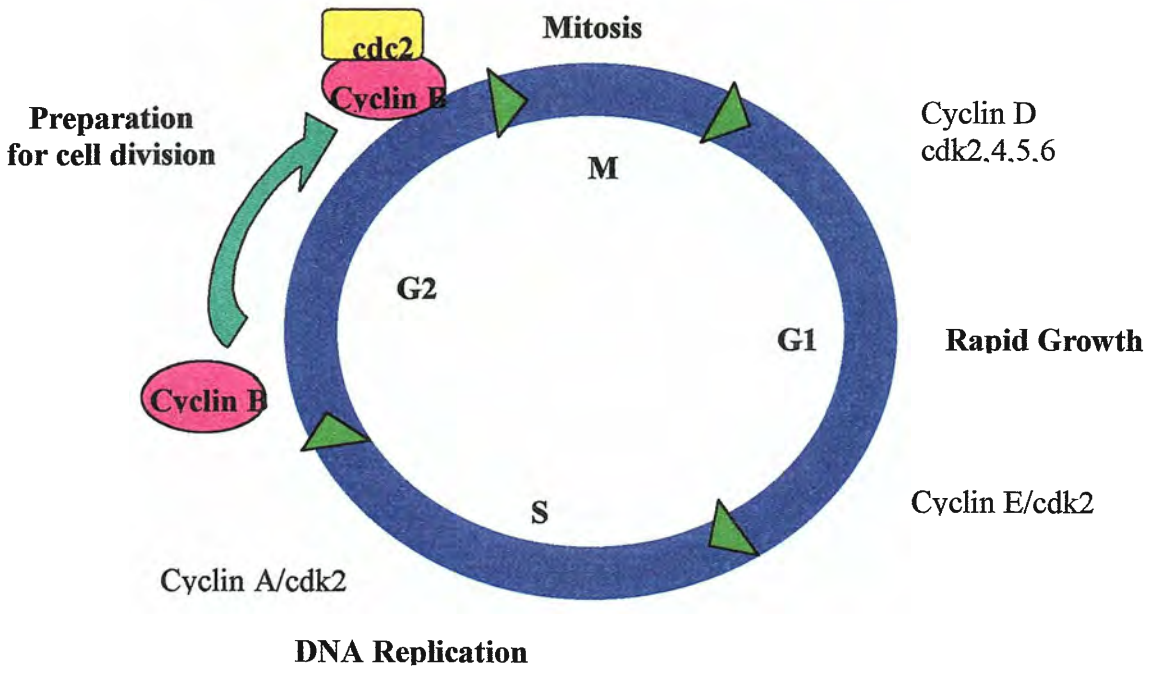

Figure 2. Major Events in Cell Cycle

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Apigenin treatment resulted in an inhibition of both p34 cdc2 kinase activity and a decrease in cyclin B protein levels. Therefore, these findings suggest that apigenin may act as a chemopreventive agent by blocking aberrant cell cycle and proliferation. Moreover, apigenin treatment (80μM) for 48 hr effectively blocked the cell cycle at G_2/M in SW480 cells (64% of cells in G_2/M), while having a more modest effect in the HT29 (42%) and Caco2 (26%) cells. The different effectiveness of apigenin on cell growth and cell cycle arrest in these three cell lines was suggested to be related, in part, to the different genetic background of these cells. Thus, it lead to the implication that apigenin may have a stronger effect on tumors with certain genetic mutations, while having limited effects in cells with wild-type genes.

Colon Cancer

Carcinogenesis is characterized by the loss of normal control and the loss of a balance of proliferation, maturation and apoptosis in cells (Kinzler and Vogelstein 1996)(see Figure 3). Initiation of carcinogenesis may be triggered by DNA damage in the cells, resulting in gene mutations unless the genetic damage is corrected. These mutations may affect two types of genes that are involved in cellular growth and differentiation: oncogenes and tumor suppressor genes (Kinzler and Vogelstein 1996). Oncogenes are mutated versions of normal cellular genes (proto-oncogenes) that regulate proliferation and differentiation pathways (Allen 1995). Expression of an oncogene results in gain-of-function in a cellular process, enhancing cell proliferation or facilitating apoptosis, such as cHaRas (Allen 1995). On the other hand, tumor suppressor genes such as adenomatous polyposis coli (APC) or p53

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typically inhibit cellular proliferation. Mutations of the tumor suppressor genes, resulting in a loss in the regulated growth inhibition, will facilitate and enhance proliferation (Morin et al. 1996). Studies have also determined another type of gene involved in carcinogenesis: DNA-damage genes. These genes are involved in the repair of damage in order to maintain the integrity of the genome. Mutations in these genes may lead to genetic instability, which are also found in many tumors (Allen 1995).

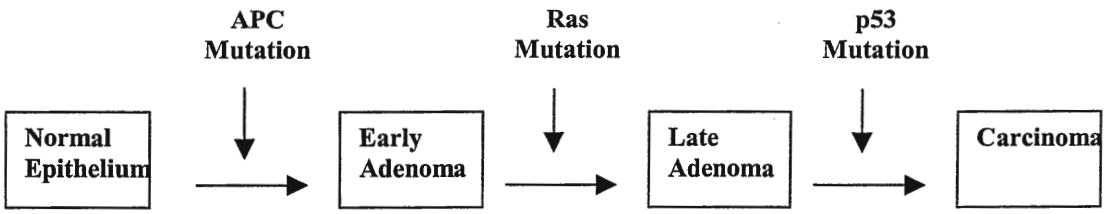

Figure 3. Vogelstein Model of Colon Carcinogenesis

The primary functional cell of the intestinal mucosa is the enterocyte. It is produced by a stem cell in the colonic crypt and migrates (when mature) towards the top of the crypt (Goss and Groden 2000). There, it reaches senescence and is shed into the lumen of the bowel. An imbalance in the homeostasis between proliferation and apoptosis of the enterocytes can result in colon carcinogenesis. Pathological outgrowths of normal developing colonic crypts give rise to the formation of polyps (White 1997). Adenomatous polyps are precursors of colon carcinomas. This relationship was established by studies of an inherited disorder, Familial Adenomatous Polyposis (FAP) (Su et al. 1992). FAP is an autosomal-dominant precancerous condition characterized by the appearance of hundreds to

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thousands of adenomatous polyps throughout the entire colorectum. The disease is caused by a germline mutation in the APC tumor suppressor gene localized on chromosome 5q21 (Kinzler and Vogelstein 1996). If left untreated, these polyps would give rise to one or more colorectal carcinomas by the third or fourth decade of life (White 1999).

The fundamental cause of colorectal cancer resides in alterations or mutations of genes involved in cellular growth and differentiation (Kinzler and Vogelstein 1996). During the development of colorectal cancer, one of the early events involved in colorectal tumorigenesis is associated with mutations in or inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene. The APC gene is presumably involved in a broad spectrum of cellular functions as well as playing a key role in the maintanace of homeostasis in continuously proliferating tissues, and preventing uncontrolled growth and tumor formation (Kinzler and Vogelstein 1996). APC gene, isolated in 1991, was localized to human chromosome 5q21 and encodes for a large protein of 2,843 amino acids (Kinzler et al. 1991). It contains 16 exons (15 coding and 1 non-coding), the largest (exon 15) containing over 75% of the 8,535 base pairs of coding sequence. Exon 15 is the target of most germline mutations in FAP and somatic mutations in tumors (Nishisho et al. 1991). An important function of the APC protein is to regulate free β-catenin level through the cooperation with glycogen synthase kinase 3β (GSK-3β) (Kinzler and Vogelstein 1996). β-catenin is a cytoplasmic protein, which has important functions in cellular adhesion and development. β-catenin is associated with cadherins, which are transmembrane proteins responsible for cell-cell interactions of the epithelial cells (Su et al. 1993). The APC protein contains a unique motif for β-catenin binding, consisting of a 15-amino acid sequence binding sites (Su et al. 1993). Moreover, the APC also contain 20-amino acid sequence repeats, which require

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phosphorylation by (GSK3β) in order to bind β-catenin (Rubeinfeld et al. 1996). When free β-catenin levels are high in the cytoplasm, GSK3β associates with the APC-β-catenin complex, forming a multi-protein complex (Kinzler and Vogelstein 1996). β-catenin is then phosphorylated, which leads to its disassociation from the complex and its degradation through an ubiquitin-dependant proteasomal pathway (Aberle et al. 1997). Together, these observations suggest that APC and GSK3β may function together to control β-catenin levels in the cytoplasm (see Figure 4).

APC appears to be a unique microtubule associated protein, as it has one domain that directly binds to the microtubules involved in cell migration (Mimori-Kiyosue 2000). It moves along the microtubules and concentrates on their growing ends, actively contributing to the assembly and reorganization of the microtubule networking. An adjacent binding domain on the APC protein binds to EB1, a member of the family of tubulin-binding proteins that interact with the microtubules (Su et al. 1995). These findings suggest that APC is involved in the process of cell migration and cell division. APC may also play an important role in regulating cell adhesion. As mentioned before, APC binds and regulates β-catenin, an intracellular protein involved in cell-cell adhesion between epithelial cells through interactions with cadherins (Su et al. 1993). APC does not directly associate with the cadherins, but only through association with β-catenin proteins.

APC and β-catenin are involved in the wnt-1 signaling pathway (Akiyama 2000) (Figure 4). The wnt-1 protein is involved in growth and developmental signaling pathways in cells. When wnt-1 binds to its transmembrane receptor frizzled, it activates a signal transduction pathway involving a protein called disheveled (dsh), which impacts negatively on GSK3β (Goss and Groden 2000). Repression of GSK3β will cause the inability of APC

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to bind to β-catenin, resulting in the repression of cytoplasmic β-catenin degradation. Therefore, the major outcome of wnt-1 signaling is the stabilization of β-catenin and its accumulation in the cytoplasm.

APC gene was found to be a critical element in the colon cancer pathway as more than 80% of colorectal tumors have inactivating APC gene mutations (Goss and Groden 2000). APC mutations lead to the formation of an adenoma, which are precursors of carcinomas as they are formed as pathologic outgrowths of a normal developing colonic crypt (Kinzler and Vogelstein 1996, White 1999). Most mutations found in the APC gene are either frameshift mutations or nonsense point mutations (Laurent-Puig et al. 1998, Nagase and Nakamura 1993). Most of these mutations lead to a stop codon in the APC reading frame, resulting in the premature termination of the polypeptide chain and the production of a truncated protein (Kinzler and Vogelstein 1996). In the absence of functional APC, the wnt-1 pathway is turned on (Akiyama 2000). As mentioned earlier, the activation of wnt-1 leads to the accumulation of free β-catenin in the cytoplasm. Free β-catenin is translocated to the nucleus where it associates with the T-cell transcriptional factors (Tcf) or lymphoid enhancer factor (Lef) transcription factors. The β-catenin /Tcf or Lef complex may modulate the transcription of Tcf/Lef-regulated target gene expression, such as c-myc. (He et al. 1998).

The c-myc proto-oncogene is a member of the myc family of genes, which are assumed to be involved in the regulation of cell proliferation, differentiation and apoptosis (Fearon and Vogelstein 1990). The c-myc induction of either the apoptotic pathway or the proliferative pathway is dependent upon the corresponding signaling of survival factors or cell death factors (Yokota et al. 1986). Only those cells receiving the appropriate survival

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signaling would continue to grow and undergo c-myc-induced cell proliferation. The c-myc-induced apoptotic pathway is activated through a single genetic lesion in cells, resulting in apoptosis instead of proliferation. However, deregulation of the c-myc expression, leading to uncontrolled cell proliferation, was found in 60% to 80% of the human colorectal cancers (Goh et al. 1996). He et al. (1998) explained this phenomenon through studies involving the identification of cancer-promoting genes at the end of the wnt-1 pathway. c-myc was

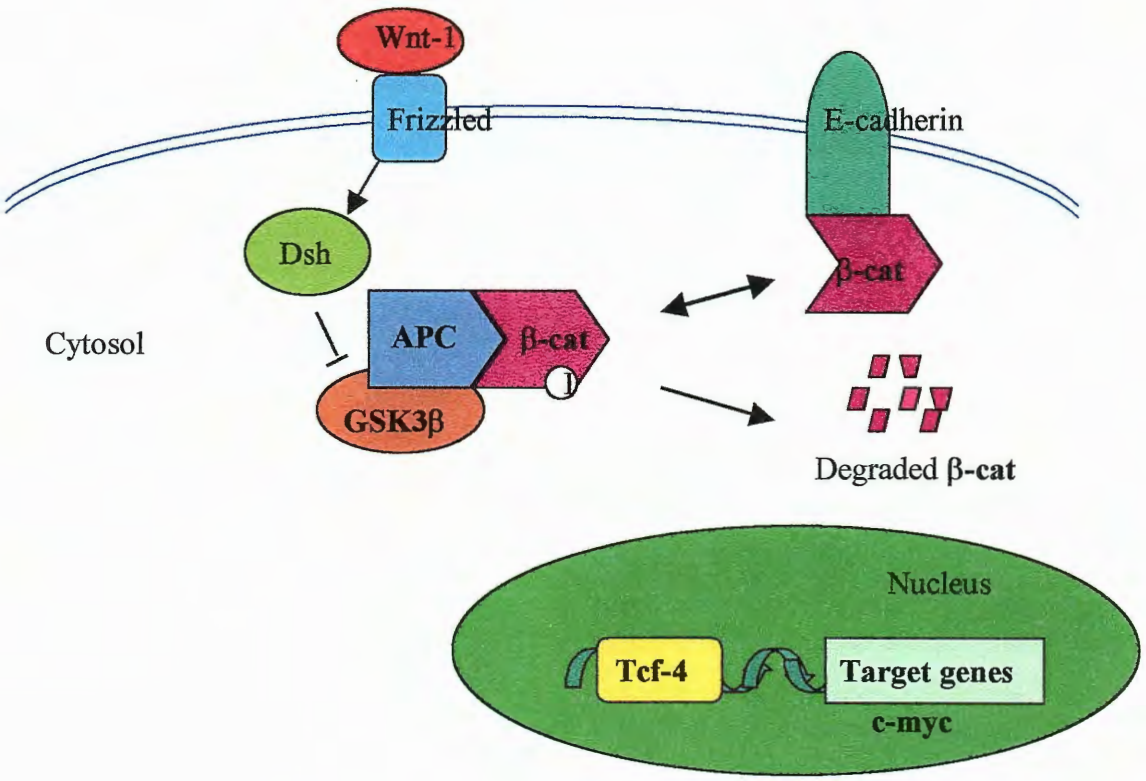

Figure 4. APC in Normal Cells

identified as one of the Tcf-regulated genes involved in the wnt-1 signaling pathway, as they found that c-myc expression was strongly suppressed by the induction of wild-type APC expression. Their findings suggest that the APC protein may regulate c-myc expression through modulating β-catenin levels, thus controlling the β-catenin/Tcf target c-myc gene transcription.

Colon Cancer Models

The study of colon carcinogenesis involves the application of a wide variety of model systems, thus includes cell culture and animal models. Many animal models have been developed to simulate the conditions of various human diseases. Reliable animal models are critical as they are valuable in understanding human diseases. An example can be given by the azoxymethane (AOM) model system. AOM is a potent inducer of carcinomas of the large intestine in various strains of male and female rats (Reddy and Maruyama 1986). The AOM treatment induces colon tumors predominantely in the distal colon, which is similar to the distribution of tumors in the human colon (Holt et al. 1996). Additionally, as described below, mutations induced by AOM treatment are similar to the mutations found in human colon tumors. Mutations in the tumor suppressor gene APC are known to be early events in the colon cancer process in humans. Studies by Maltzman et al. (1997) demonstrated that AOM-induced mouse colon adenomas and adenocarcinomas did not express full-length APC proteins,in agreement with observations in human colon carcinogenesis. Other mutations induced by AOM treatment in rodents similar to human colon tumors also include ras oncogene mutations (at codon 12 of K- and H-ras) (Singh et al. 1994).

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Transgenic animal models such as the multiple intestinal neoplasia (Min) mouse model were also developed and are used predominantly to study the relationship between the nutritional factors and colon carcinogenesis. The Min mouse carries a fully penetrated dominant mutation at codon 850 of the murine APC gene, resulting in a truncated APC protein (Su et al. 1992). Several studies using Min mice demonstrated that ~97% of tumors were found in the small intestine and ~3% of the tumors were in the colon, all the tumors being adenomas (Jacoby et al. 1996). Although the Min mice have an autosomal dominant inherited predisposition to develop numerous intestinal tumors, it appears that the phenotypic expression of APC mutations in the Min mouse is somewhat different from human colon cancer. Patients with Familial Adenomatous Polyposis (FAP) develop adenomas found exclusively in the colon and some in the duodenum, whereas the Min mouse develop the majority of the adenomas in the small intestine (Su et al. 1992).

The animal models mentioned above are useful in understanding the relationship between nutritional factors and colon cancer. The identification of the mechanisms where diet may alter cancer is important in optimizing intakes of foods and nutrients for cancer prevention in animals and later in the application to human diets. Therefore, results from studies using both genetic based and chemically-induced models may contribute significantly to many aspects in the understanding of genetics and nutritional factors in relation to colon carcinogenesis. However, the integration of cell culture studies and experimental animal studies would provide a much better understanding in the relationship between nutrition and cancer. Many cell model systems are presently used to solve enigmatic events in carcinogenesis, and to determine the mechanisms behind the modulation of dietary factors in cancer prevention. Cell culture studies involving different cancer cell lines help contribute to

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the simulation and better optimization of certain physiological conditions. In addition, the application of a particular cell model system may prove useful as a basis for testing the hypothesis of a particular study. For example, the development of a particular cell model system involving transfected human colon cancer cell lines, allow the induction of exogenous factors, which may mimic a "normal" cellular environment. An example of the application of this particular cell model system will be presented further on in this present study.

HT29-APC and HT29-GAL Cell Culture Model

In this study, we used the cell model system consisted of transfected HT29 cells (HT29-APC and HT29-GAL), which proved useful as we aimed to determine the impact of the APC tumor suppressor gene on the effectiveness of apigenin to inhibit tumor cell growth. The HT29 is a human colon cancer cell line that contains APC mutations, p53 gene mutations and wild-type ras (Huang et al. 1994). The transfected HT29-APC contains a $ZnCl_2$-inducible APC gene under control of the metallothionine promoter, whereas the HT29-GAL cells, containing β-galactosidase plasmid, served as the control (Morin et al., 1996). Previous studies by Morin et al. (1996) demonstrated that the expression of the wild-type APC in the HT29-APC cells (following $ZnCl_2$ treatment) induced an increase in apoptosis determined by TUNEL assay. Western blot analysis determined the expression of full-length APC protein in the HT29-APC cells after they were treated with 100μM of $ZnCl_2$ (Morin et al. 1996). It was proposed by He et al. (1998) that the APC protein prevented β-catenin from forming a complex with Tcf-4, which consequently supresses c-myc transcription. As mentioned earlier, the over-expression of c-myc accompanied by uncontrolled proliferation in the

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colonic tumor cells was suppressed in the HT29-APC cells expressing full-length APC protein following $ZnCl_2$ treatment (He et al. 1998).

Hypothesis

The hypothesis of this present study was that apigenin would induce a G_2/M cell cycle arrest in colon cancer cells with APC mutations. We proposed that apigenin would be more effective in cellular growth inhibition in the zinc-treated HT29-GAL cells than in the zinc-treated HT29-APC cells, since the HT29-GAL cells do not express the wild-type APC following $ZnCl_2$ treatment. Cell growth inhibition in the HT29-GAL cells was hypothesized to be due to the apigenin-induced G_2/M cell cycle arrest. On the other hand, we believe that the wild-type APC expression (following $ZnCl_2$ treatment) in the HT29-APC cells would prevent apigenin-induction of G2/M cell cycle arrest. The HT29-APC cells would also show a decrease in cell number upon treatment with apigenin and $ZnCl_2$. However, the decrease in cell number would be due to the expected increase in apoptosis following $ZnCl_2$ treatment (induction of wild-type APC expression).

Objective

Our main objective is to test our hypothesis utilizing the cell model system involving transfected HT29 (HT29-APC and HT29-GAL) cells described earlier. Cell growth, as well as flow cytometry results, revealed the effect of apigenin on cell proliferation and cell cycle. TUNEL assay was conducted to demonstrate the induction of apoptotic cells following $ZnCl_2$

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treatment (induction of wild-type APC) in HT29-APC cells. The ability to restore the expression of the wild-type APC in the HT29-APC cells (following $ZnCl_2$ treatment) allowed us to investigate the importance of the APC mutations found in human cancer cells for apigenin-induced G_2/M cell cycle arrest. However, the induction of the wild-type APC expression in the $ZnCl_2$-induced HT29-APC cells did not correct the other existing mutations (the APC and p53 mutations), as these cells are derived from the HT29 colon cancer cells.

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CHAPTER 2. IMPACT OF ADENOMATOUS POLYPOSIS COLI (APC) TUMOR SUPRESSOR GENE IN HUMAN COLON CANCER CELL LINES ON CELL CYCLE ARREST BY APIGENIN

A paper to be submitted to the journal Carcinogenesis

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Abstract

Apigenin is a dietary flavonoid that is widely distributed in fruits and vegetables. It was previously shown to induce G_2/M cell cycle arrest in three different human colon cancer cell lines that have APC mutations (SW480, HT29 and CaCo2). The research reported here assessed the importance of the APC tumor suppressor mutation in relation to the ability of apigenin to induce cell cycle arrest. The cell model system utilized for this study consisted of HT29-APC cells, which contain an inducible wild-type APC gene under control of the metallothionine promoter. Induced wild-type APC expression was characterized by an increase in apoptosis in cultured HT29-APC cells treated with 100μM $ZnCl_2$. HT29-GAL cells, containing β-galactosidase plasmid, served as the control. Treatment with apigenin alone (from 0μM to 80μM) for 48 hours resulted in a significant ($P<0.05$) reduction in the cell number concurrent with flow cytometry results showing a dose-dependent accumulation of cells in G_2/M phase in both uninduced HT29-APC and HT29-GAL cells. Upon induction of the APC gene in HT29-APC cells, Terminal Deoxytransferase-Mediated Deoxyuridine NickEnd-Labeling (TUNEL) assay results showed that the percentage of apoptotic HT29-

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APC cells treated with 100μM of $ZnCl_2$ and 0μM apigenin increased significantly ($P<0.05$) after 120hr treatment. Additionally, apigenin treatment (80μM) appeared to enhance the sensitivity of the HT29-APC cells to apoptosis when treated with $ZnCl_2$ to induce wild-type APC expression. On the other hand, no significant changes in the percentage of apoptotic cells were present either in the HT29-APC cells when treated with apigenin alone or in the HT29-GAL cells when treated with either apigenin and/or $ZnCl_2$. Flow cytometric analysis showed an increase ($P<0.05$) in the percentage of cells in G2/M when HT29-APC cells were treated with 80μM apigenin for 120 hr. This increase was not present in HT29-APC cells when treated with both 80μM apigenin and 100μM $ZnCl_2$ for 120hr. Nevertheless, the apigenin-treated HT29-GAL cells showed a significant ($p<0.05$) accumulation of cells in G_2/M regardless of the presence of $ZnCl_2$. These results suggest that APC dysfunction may be critical for apigenin to induce cell cycle arrest in human colon cancer cell lines.

Introduction

A diet rich in fruits and vegetables has been associated with a reduced incidence of cancers, including colon cancer (Slattery et al. 1997). Several epidemiological studies have indicated that the higher consumption of fruits and vegetables may lower the risk of certain cancers (Williams et al. 1999). Numerous classes of compounds present in fruits and vegetables are assumed to take the role of cancer-preventive agents (Birt et al. in press, Hill 1999). Among these, one class of compounds includes the flavonoids, which are widely distributed among plants. Apigenin [5,7,4'-trihydroxyflavone], a common dietary flavonoid, has been found in a variety of fruits and vegetables including parsley, onions, apples, tea, and

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chamomile. It has been shown to induce cell cycle arrest in both epidermal and fibroblast cells (Lepley et al. 1996), and to inhibit skin tumorigenesis in murine model (Wei et al. 1990).

Our laboratory has previously studied the possible role of apigenin as a chemopreventive agent in colon carcinogenesis. Animal studies revealed that colonic ODC activity and ACF formation in CF-1 mice were reduced with 0.1% dietary apigenin when compared with the control (Au, et al. unpublished). Wang et al. (2000) has shown that apigenin induced G_2/M cell cycle arrest in three different colon cancer cell lines with truncated APC genes (SW480, HT29, and Caco2). The different effectiveness of apigenin on cell growth and cell cycle arrest in these three cell lines was suggested to be related, in part, to the different genetic background of these cells. Thus, it lead to the implication that apigenin may have a stronger effect on tumors with certain genetic mutations.

The cause of colorectal cancer is now widely accepted to be the accumulation of mutations in specific genes controlling cell division, apoptosis and DNA repair. One of the early events involved in colorectal tumorigenesis is associated with mutations in or inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene. The APC gene is presumably involved in a broad spectrum of cellular functions as well as playing a key role in the maintanace of homeostasis in continuously proliferating tissues, and preventing uncontrolled growth and tumor formation (Kinzler and Vogelstein 1996). It was found to be a critical element in the colon cancer pathway as more than 80% of colorectal tumors have inactivating APC gene mutations (Goss and Groden 2000). In the absence of functional APC, free β-catenin accumulates in the cytoplasm, leading to its translocation to the nucleus. β-catenin is a cytoplasmic protein, which has important functions in cellular adhesion and

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development (Morin et al. 1996). It associates with DNA-binding protein T-cell transcriptional factors (Tcf) and this β-catenin /Tcf complex may modulate the transcription of target genes such as c-myc (He et al. 1998). Activation of the signaling pathway involving the proto-oncogene c-myc will lead to the promotion cell growth and proliferation. c-myc expression levels are usually increased in most colorectal cancers and may be linked as a direct consequence of mutations of the APC tumor suppressor gene (He et al.1998).

To address the hypothesis that APC mutations are critical for apigenin to induce a G_2/M cell cycle arrest in human colon cancer cell lines, a unique cell model system was used, consisting of transfected HT29 (HT29-APC and HT29-GAL) human colon cancer cell lines. The HT29 cells are known to have APC gene mutations (frameshift or nonsense point mutations) on chromosome 5q21 (Kinzler and Vogelstein 1996), in addition to a mutated p53 gene and a wild-type ras gene (Huang et al. 1994). Transfected HT29-APC cells contain an inducible wild-type APC gene following addition of 100μM $ZnCl_2$. This concentration of $ZnCl_2$ was previously shown by Morin et al. (1996) to induce wild-type APC expression in the HT29-APC cells determined by Western Blot analysis detecting full-length APC protein in the HT29-APC cells following $ZnCl_2$ treatment. The wild-type APC expression in HT29-APC cells (following addition of 100μM $ZnCl_2$) was manifested by an increase in apoptosis determined by TUNEL assay (Morin et al. 1996). On the other hand, transfected HT29-GAL cells contain a galactosidase plasmid, serving as a control cell line. We performed TUNEL assay to determine the induction of apoptosis following $ZnCl_2$ treatment in apigenin-treated cells. In addition, cell count and flow cytometric analysis were performed to investigate the influence of apigenin on cell growth and cell cycle in these cells.

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Materials and Methods

Cell Number and Cell Growth

HT29-APC and HT29-GAL cells were obtained from Dr. Bert Vogelstein's laboratory (Morin et al. 1996). Cells were cultured in McCoy's 5A Modified Media (Sigma) with 10% Fetal Bovine Serum. They were maintained in an incubator at 37°C with 95% atmospheric O_2 and 5% CO_2. Cells were plated and treated when they reached 50-60% confluency.

For cell growth, cells were treated with different doses of apigenin and in the presence or absence of 100μM of $ZnCl_2$. Cells were harvested (at time points indicated in the results) by trypsinization for 10 minutes. The number of cells was counted with a hemacytometer. All four 1-mm corner squares of the hemacytometer were counted and the average was calculated.

Cell Cycle Analysis

Cells were treated with apigenin and/or with $ZnCl_2$ (100μM) and harvested by trypsinization. Cells were fixed in 70% ethanol and stored at 4 °C overnight. Fixed cells were prepared for flow cytometry analysis by washing twice with Phosphate Buffer Saline Solution (PBS), pH 7.4. Cells were resuspended in propidium iodide (20-mg/ml) staining solution containing 1μg/ml RNase in 10 mM PBS, pH 7.45 and incubated at 37 °C for 30 minutes. Flow cytometry analysis was performed with a FACStar-plus flow cytometer (Becton Dickinson) with an excitation at 488 nm and an emission at 630 nm.

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Terminal Deoxytransferase-Mediated Deoxyuridine NickEnd-Labeling (TUNEL) Assay

Cells were cultured in the presence or absence of 100μM $ZnCl_2$ along with apigenin treatment at 0μM and 80μM. Cells were harvested by trypsinization following 0, 48 and 120 hours of treatment. Cells were initially fixed for 30 min using 1% paraformaldehyde, followed by secondary fixation in 70% (v/v) ethanol and stored at -20°C overnight. Fixed cells were then prepared for TUNEL assay analysis using the APO-BRDU™ Kit (Pharmigen). This involved incubating the cells initially in DNA labeling solution for 1hr followed by 30 min-incubation periods with the Antibody Solution and PI/Rnase A Solution. Stained cells were then analyzed by flow cytometry with a FACStar-plus flow cytometer (Becton Dickinson) with an excitation at 488 nm and an emission at 630 nm.

Statistics

Results were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the SPSS (Version 10.0) statistical program. Comparisons across cell lines were analyzed by comparing individual means using Student's t-test. Data including treatments with apigenin and zinc from HT29-APC were analyzed separately from HT29-GAL, using analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

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Results

Impact of Apigenin on Cell Growth and Cell Cycle in Uninduced Cells

Treatment of the HT29-APC and HT29-GAL cells with apigenin doses up to 80μM resulted in a dose-dependent decrease in cell number compared to the vehicle control (Figure 1). Apigenin treatment at 60 and 80μM for 48 hours significantly ($p<0.05$) decreased in the number of both HT29-APC and HT29-GAL cells. Both HT29-APC and HT29-GAL cells showed a significant ($p<0.05$) increase of cells in the G_2/M phase by flow cytometric analysis after apigenin treatment at 80μM for 48 hours (Figure 2). The decrease in the percentage of cells in G1 phase at 60 and 80μM apigenin treatment ($p<0.05$) was associated with the increase in the percentage of cells in the G_2/M phase. The percentage of cells in S phase did not change with apigenin treatment.

Impact of Apigenin on Cell Growth and Cell Cycle Arrest in Induced Cells

The 0μM apigenin-treated HT29-GAL cells showed a significant ($p<0.05$) increase in cell number at 120hr treatment (Figure 3). This increase was also observed in the HT29-GAL cells when incubated with $ZnCl_2$. On the other hand, the HT29-APC cells did not show a significant increase in cell number when treated with $ZnCl_2$ compared to the significant ($p<0.05$) increase when treated with 0μM apigenin for 120 hours. Both cell lines showed a significant ($p<0.05$) decrease in cell number when treated with apigenin alone (80μM) for 48 and 120 hours. However, the decrease in cell number with treatment at 80μM

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apigenin+100μM $ZnCl_2$ was more striking than the decrease in HT29-GAL cells treated with 80μM apigenin+100μM $ZnCl_2$ over time ($P<0.03$).

Flow cytometric analysis was assessed for cells treated with either apigenin alone or in the presence of 100μM $ZnCl_2$. The HT29-APC cells showed a significant accumulation of cells at G_2/M after treatment of 80μM apigenin for 48 and 120 hours. However, when treated with $ZnCl_2$, the HT29-APC cells did not show an increase in cells in G_2/M with 80μM apigenin (Figure 4). In contrast, the HT29-GAL cells showed a significant accumulation of cells in G_2/M phase after apigenin treatment regardless of the presence of $ZnCl_2$.

Induction of Apoptosis (upon addition of $ZnCl_2$) on Apigenin-Treated HT29-APC and HT29-GAL cells

The HT29-GAL cells treated with either apigenin and/or $ZnCl_2$ did not show a significant change in the percentage of apoptotic cells (Figure 5). Treatment of HT29-APC cells with 100μM of $ZnCl_2$ in the presence or absence of apigenin resulted in a significant increase in the percentage of apoptotic cells (suggesting the wild-type APC expression). The percentage of apoptotic HT29-APC cells treated with 0μM apigenin + 100μM of $ZnCl_2$ increased significantly after 48 and 120hr (refer to Appendix of example of TUNEL assay results). Interestingly, treatment of HT29-APC cells with 80μM apigenin + 100μM of $ZnCl_2$ resulted in a more pronounced increase of apoptotic cells ($p<0.05$) compared to 0μM apigenin + 100μM of $ZnCl_2$ treatment.

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Discussion

Several epidemiological studies have found that a diet with abundant fruits and vegetables is strongly correlated to a reduced risk of cancers (Williams et al. 1999). Suggested dietary constituents have included a variety of compounds, including the flavonoids. Apigenin, a plant flavonoid, has been a main focus in our laboratory as a potential agent for chemoprevention of colon carcinogenesis, both in vivo and in vitro. Previous studies in our laboratory have suggested that apigenin may have a stronger effect on tumors with certain genetic mutations (Wang et al. 2000). This present study investigated the efficacy of apigenin on colon cancer cell lines with different mutational spectra using transfected HT29 (HT29-APC and HT29-GAL) human colon cancer cells. HT29-APC cells contain an inducible APC gene, and the HT29-GAL cells served as the control. In order to assess an expected outcome of APC expression in the HT29-APC cells, we performed the TUNEL assay following treatment of cells with 100μM of $ZnCl_2$. Our results showed that the presence of 100μM of $ZnCl_2$ increased apoptosis in HT29-APC cells, while the HT29-GAL cells demonstrated no change in response to zinc treatment. These observations were in agreement with Morin et al. (1996) that an increase in apoptotic cells (determined by TUNEL assay) was revealed in zinc-induced HT29-APC cells, suggesting the expression of APC.

Apigenin treatment had a strong growth inhibitory effect on both HT29-APC and HT29-GAL. As expected, both cell lines showed a significant decrease in cell growth after apigenin treatment for 48 hours, which may be correlated with the apigenin-induced G_2/M cell cycle arrest. These results were consistent with previous observations by Wang et al.

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(2000) in which apigenin inhibited the growth of three different colonic carcinoma cell lines by blocking the cell cycle at G_2/M phase. The three different cell lines all had APC mutations, consistent with the cell lines used in the present study. Since the transfected HT29-APC and HT29-GAL cells are derived from the HT29 colorectal cancer cell line, they contain APC gene mutations (frameshift or nonsense point mutations) on chromosome 5q21 (Kinzler and Vogelstein 1996), in addition to a mutated p53 gene and a wild-type ras gene (Huang et al. 1994). As a consequence, the induction of the wild-type APC gene (following $ZnCl_2$ treatment) in HT29-APC did not correct the APC or p53 mutations present in the HT29 colorectal cancer cell line. However, the presence of wild-type APC following $ZnCl_2$ treatment presumably restored normal APC function and prevented apigenin induction of G_2/M cell cycle arrest in the HT29-APC cells. In contrast, in the presence of $ZnCl_2$, apigenin induced a G_2/M cell cycle arrest in the HT29-GAL control cells. As mentioned before, the presence of $ZnCl_2$ induces wild-type APC expression in the HT29-APC cells. Therefore, our data suggest that the wild-type APC expression in $ZnCl_2$-treated HT29-APC cells blocks the ability of apigenin to induce a G_2/M cell cycle arrest. These observations predict the importance of APC mutations for apigenin to induce G_2/M cell cycle arrest and thus affect cell growth.

In order to assess the effect of apigenin on the HT29-APC and HT29-GAL cells, different doses of apigenin at 0, 20, 30, 40, 60 and 80μM were used in these experiments. Our data revealed that treatment with 80μM apigenin resulted in the strongest physiological effect of apigenin on the inhibition of cell growth and induction of cell cycle arrest in both cell lines, while displaying an intermediate effect with treatments at lower doses of apigenin. Because the later experiments involved assessing the effect of apigenin in the presence or

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absence of wild-type APC gene, the number of different groups increased as it involved two different cell lines, three different time points, in addition to the different apigenin treatments. Therefore, apigenin treatments were performed at 0, 40, and 80μM apigenin (as indicated in results) in an effort to achieve optimal responses in the cell lines and simply the experimental design. Presently, no studies have reported comparisons between *in vivo* and *in vitro* apigenin concentrations at different doses of apigenin treatment in colon cancer cells. However, the cellular concentrations detected in the apigenin-treated C50 murine keratinocyte cultured cells at doses up to 45μM (Lepley et al., 1996) were similar to the concentrations revealed in the *in vivo* mouse epidermal cells treated topically with 5μM apigenin, a cancer preventive dose (Li et al. 1996). In addition, Lepley et al. (1996) demonstrated that apigenin treatment at doses up to 45μM resulted in the induction of a reversible G_2/M cell cycle arrest in C50 murine keratinocyte cells. Similarly, the apigenin-induced G_2/M cell cycle arrest at 80μM was shown to be reversible in the SW480 human colon cancer cells (Wang et al., 2000), suggesting that the apigenin effect on the cells appeared to be cytostatic rather than cytotoxic. It should be noted that flavonoids could be toxic, as studies by Birt et al (1986) have shown that topical apigenin treatment at 100μM induced the ornithine decarboxylase (ODC) activity, a tumor promotion marker, in the mouse epidermis. However, the cellular concentration with this topical dose is unknown.

As expected, an increase in cell number with incubation time was seen when the HT29-GAL cells were treated with 0μM apigenin in the presence or absence of $ZnCl_2$, since these cells did not express wild-type APC gene upon $ZnCl_2$ treatment. Although cell numbers in the HT29-APC were increased after 120 hr treatment with 0μM apigenin when

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compared to 0 hr, cell growth was not seen when these cells were treated with 0μM apigenin and $ZnCl_2$. This observed cell growth inhibition in the HT29-APC cells was apparently due to the induction of apoptosis in the HT29-APC cells following $ZnCl_2$ treatment (induction of wild-type APC expression) as shown in our TUNEL assay results. It is noteworthy that the decrease in HT29-APC cells was more prominent than the decrease in HT29-GAL cells when treated with 80μM apigenin and 100μM $ZnCl_2$. Evidently, the decrease in the HT29-GAL cells correlated with the apigenin-induced G_2/M cell cycle arrest demonstrated earlier, as the $ZnCl_2$-treated HT29-GAL cells did not reveal an increase in apoptosis. However, the HT29-APC cells were not blocked at G_2/M cell cycle phase when treated with 80μM apigenin and 100μM $ZnCl_2$. Therefore, the decrease in HT29-APC cell growth was due, in part, to the induction of apoptosis (induction of wild-type APC gene expression) following treatment with 80μM apigenin and 100μM $ZnCl_2$. Interestingly, the HT29-APC cells revealed higher percentage of apoptotic cells when treated with 80μM apigenin and $ZnCl_2$ compared to 0μM apigenin and $ZnCl_2$. This data suggest that the apigenin-treated HT29-APC cells appeared to have enhanced apoptosis induction when expressing wild-type APC (following treatment of $ZnCl_2$). It should be noted, however, that the induction of the expression of wild-type APC together with the apigenin treatment might have affected other cellular regulatory pathways in the HT29-APC cells.

The efficacy of dietary apigenin on chemoprevention of colon carcinogenesis was investigated through a dietary approach in the Min mouse model (Au et al. unpublished). Min mice have been widely used to study colon carcinogenesis because they are transfected with APC mutations in chromosome 18 (codon 850), which will enable them to

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spontaneously develop large number of intestinal polyps. These lesions are reminiscent of familial adenomatous polyposis (FAP) (Shoemaker et al. 1997). Our results suggest that apigenin might be effective in reducing the genetically-induced tumors in the Min mouse model. However, unpublished findings (Au et al.) revealed that apigenin failed to reduce the genetically-induced tumors, this result may be due to the low doses of apigenin that were used in the diet. Further, it must be noted that, unlike human colon cancer, the majority of the adenomas in the min mice were located in the small intestine, and there were none or only a few adenomas found in the colon. Finally, the apparent discrepancy between the cell culture and the min mice experiments may be explained by the different location of the APC mutation in the Min mice (chromosome 18) and in the HT29 human colon cancer cell line (chromosome 5q21). Therefore, future studies involving other mouse models that better simulate human colon tumors will be required to determine the effectiveness of apigenin in colon carcinogenesis.

In conclusion, our results show that apigenin induced cell growth inhibition by blocking cell cycle progression at G_2/M phase in the HT29-APC and HT29-GAL cells. Wild-type APC gene expression in the zinc-induced HT29-APC cells was associated with the inability of apigenin to induce a G_2/M cell cycle arrest, suggesting that apigenin may be more effective in inducing G_2/M arrest in cells with APC mutations.

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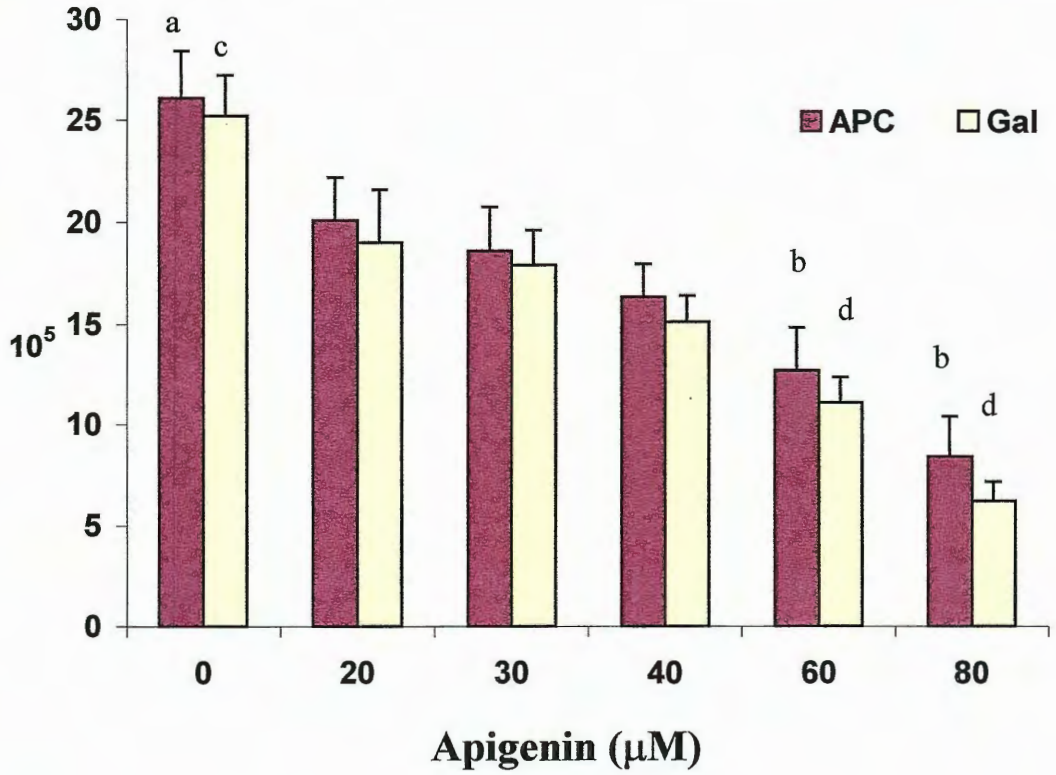

Figure1. Impact of Apigenin on HT29-APC and HT29-GAL cell numbers. Cells were treated with different doses of apigenin up to 48hr and counted using a hemocytometer (Mean± SD; n=4). Different letters within each cell line indicated a significant difference in cell count. Statistical analysis (ANOVA with Tukey's multiple comparison test) indicated a significant decrease in cell count in HT29-APC and HT29-GAL cells when treated with increamental doses of apigenin. (b<a, $P<0.05$ and d<c, $P<0.05$)

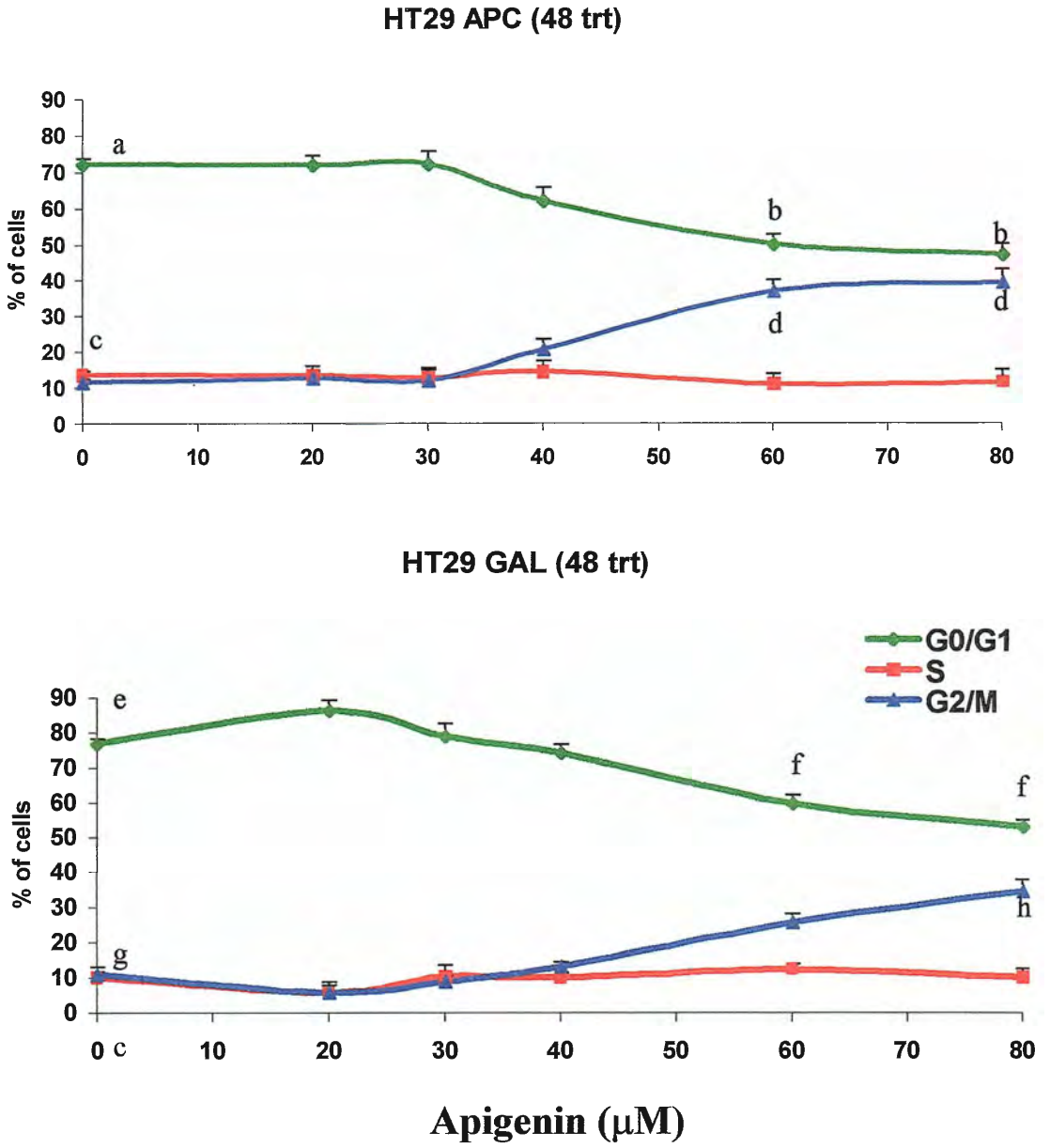

Figure 2. Impact of Apigenin on HT29-APC and HT-29-GAL cell cycle arrest. Cells were cultured in media with different doses of apigenin (0-80 μM) for 48 hours, and the cell cycle was monitored by DNA flow cytometric analysis. Means±SD from three independent experiments are shown. Different letters within each treatment indicated a significant difference in percent of G_2/M cells for each cell line. Statistical analysis (ANOVA with Tukey's multiple comparison test) indicated a significant increase in percent of G_2/M cells in HT29-APC and HT29-GAL cells when treated with increamental doses of apigenin. (a<b, $P<0.05$ and c<d, $P<0.05$)

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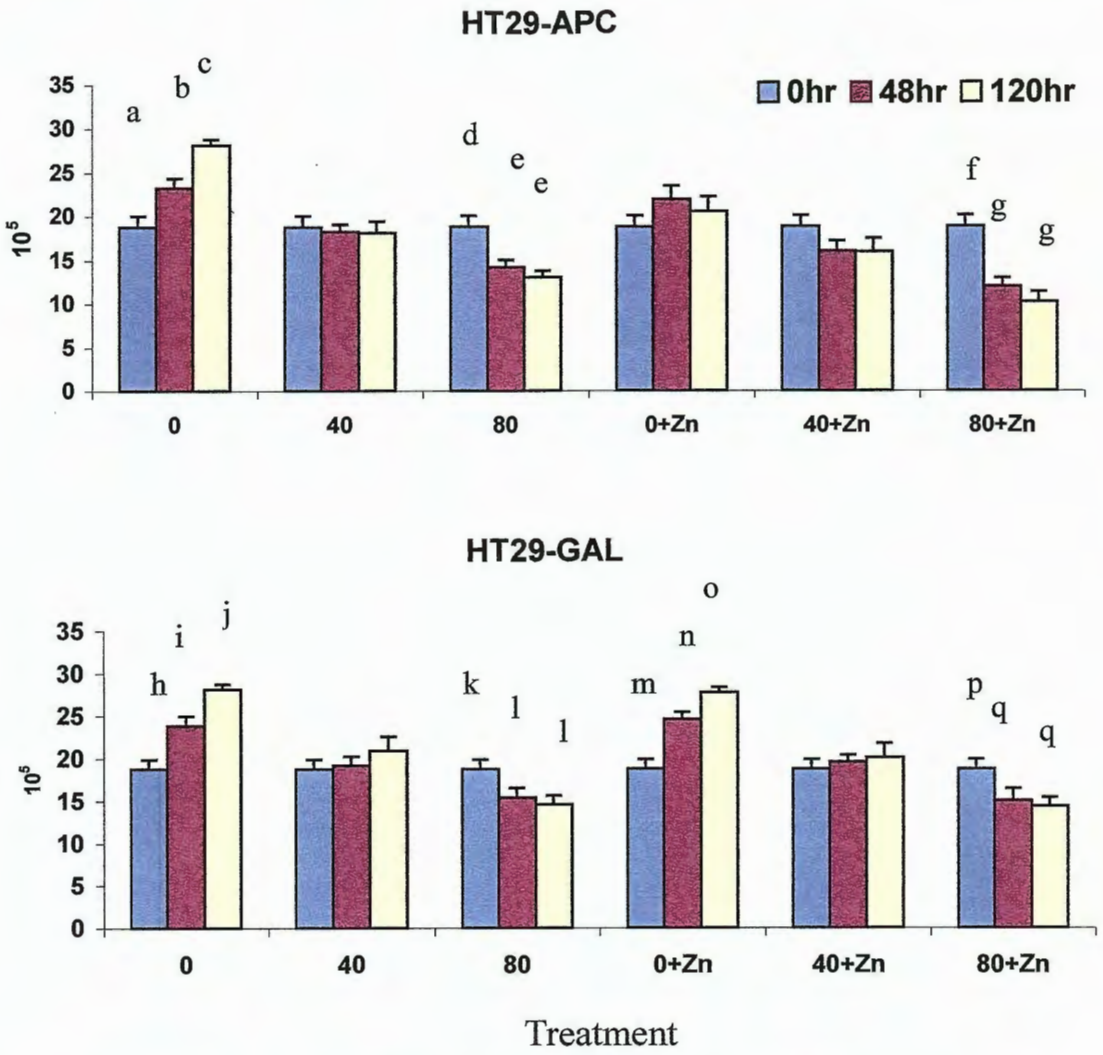

Figure 3. Impact of Apigenin and $ZnCl_2$ (induction of wild-type APC in HT29-APC) on HT29-APC and HT-29-GAL cell numbers. HT29-APC and HT29-GAL cells were cultured and treated with apigenin (0, 40 and 80μM) and/or with $ZnCl_2$ (100μM) for up to 120 hr. Means±SD from six independent experiments are shown. Data were analyzed by ANOVA with Tukey's multiple comparison test. Different letters within each treatment indicated a significant difference in cell number for each cell line. Data without letters were not significantly different between the incubation times. The slope in the HT29-APC cells (80μM+Zn treatment) was greater than the HT29-GAL cells upon similar treatment ($P<0.05$). ($a<b<c$, $P<0.05$; $e<d$, $P<0.05$; $g<f$, $P<0.05$; $h<i<j$, $P<0.05$; $l<k$, $P<0.05$; $m<n<o$, $P<0.05$; $q<p$, $P<0.05$)

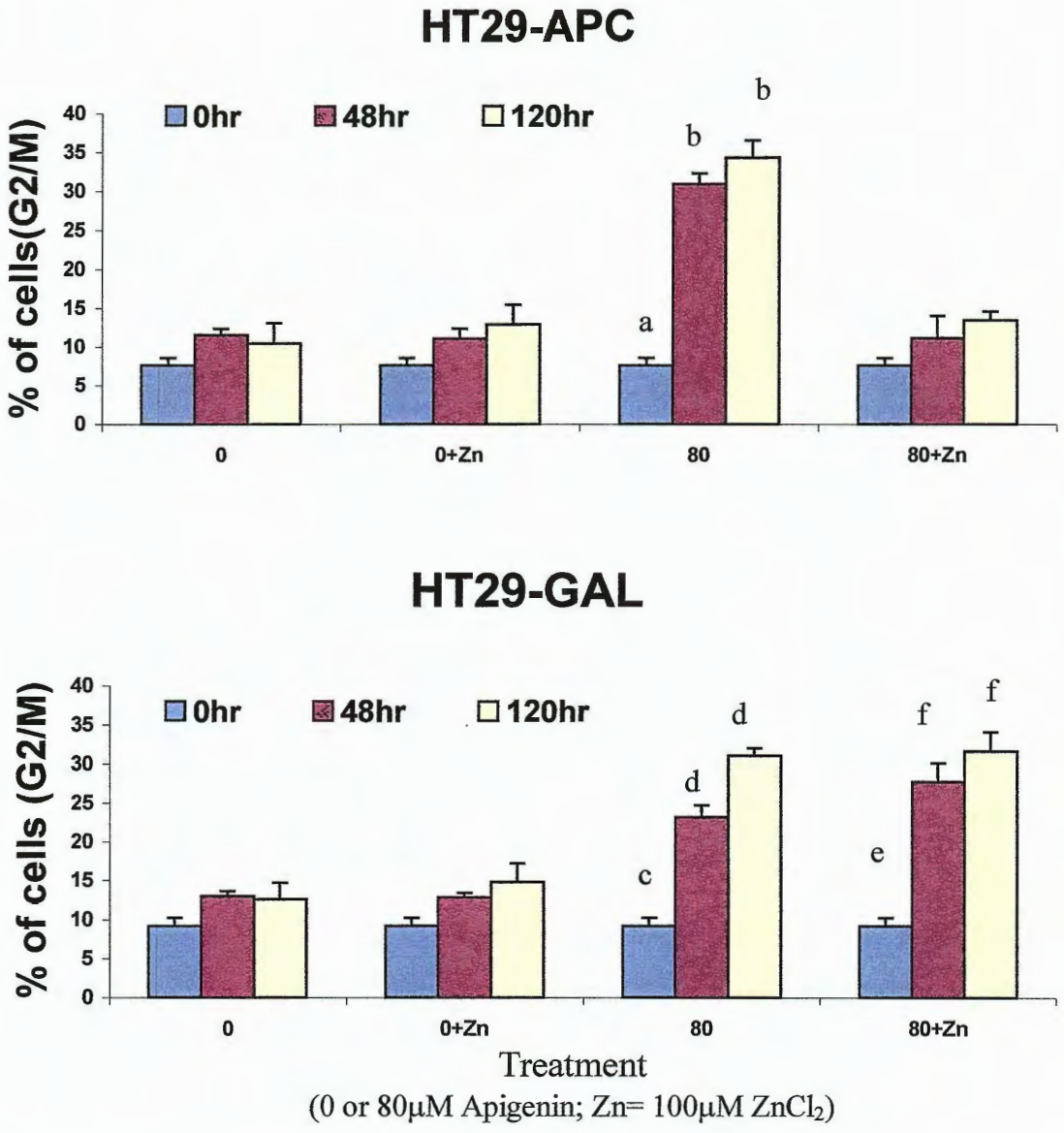

Figure 4. Impact of Apigenin and $ZnCl_2$ (induction of wild-type APC in HT29-APC) on HT29-APC and HT-29-GAL cell cycle arrest. HT29-APC and HT29-GAL cells were cultured and treated with apigenin (0 and 80μM) and/or with $ZnCl_2$ (100μM) for up to 120 hr. Means±SD from three independent experiments are shown. Data were analyzed by ANOVA with Tukey's multiple comparison test. Different letters within each treatment indicated a significant difference in percentage of G_2/M cells for each cell line. Data without letters were not significantly different between the incubation times (a<b, $P<0.05$; c<d, $P<0.05$; e<f, $P<0.05$).

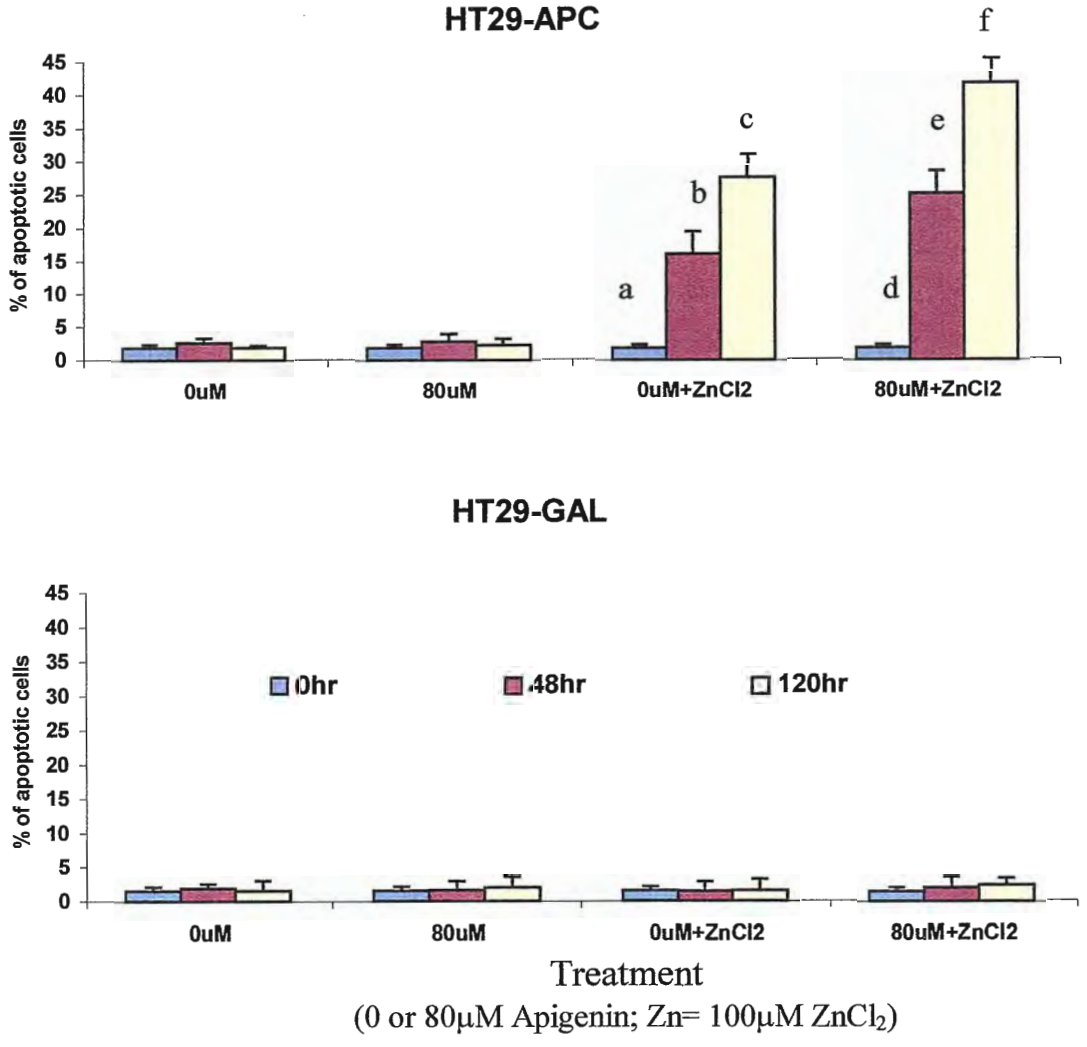

Figure 5. Induction of apoptosis (upon addition of $ZnCl_2$) on apigenin-treated HT29-APC and HT29-GAL cells. HT29-APC and HT29-GAL cells were cultured in media with apigenin (0 and 80μM) and the presence or absence of $ZnCl_2$ (100μM) for up to 120 hr. Means±SD from three independent experiments are shown. Data were analyzed by ANOVA with Tukey's multiple comparison test. Different letters within each treatment indicated a significant difference in percentage of apoptotic cells for each cell line. Data without letters were not significantly different between the incubation times. The HT29-APC cells treated with 80μM+ $ZnCl_2$ presented a greater slope compared to 0μM $ZnCl_2$ treatment ($P<0.03$). ($a<b<c$, $P<0.05$; $d<e<f$, $P<0.05$).

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CHAPTER 3. GENERAL CONCLUSIONS

This study investigated the importance of the APC tumor suppressor gene mutations in relation to the effectiveness of apigenin in inducing a G2/M cell cycle arrest in human colon cancer cells. The hypothesis of this study was that apigenin would induce a G_2/M cell cycle arrest in colon cancer cells with APC mutations. This hypothesis was proposed based on the implication that apigenin may have a stronger effect on tumors with certain genetic mutations (Wang et al., 2000). In order to test this hypothesis, we used the cell model system consisting of transfected HT29 (HT29-APC and HT29-GAL) human colon cancer cell lines mentioned earlier in Chapter one. The rationale behind using the cell model system in this study was that it allowed the induction of the wild-type APC gene expression (with the addition of 100μM $ZnCl_2$) in the HT29-APC cells, normalizing the function of the full-length APC protein expression in HT29-APC cells verified by western blot analysis (Morin et al. 1996). The HT29-GAL cells served as the control, containing a galactosidase plasmid. Thus, these cells would allow us to determine the effect of apigenin in the presence or absence of the inducible wild-type APC gene. Our data demonstrated in Chapter two confirmed our proposed hypothesis, where apigenin induced a strong inhibitory effect on the HT29-GAL cells in presence or absence of $ZnCl_2$ treatment. On the other hand, the presence of the wild-type APC gene (following treatment with $ZnCl_2$) in the apigenin-treated HT29-APC cells appeared to block the effect of apigenin, which was anticipated in our hypothesis.

Previously, He et al. (1998) used this cell model system to identify the Tcf-regulated genes involved in the wnt-1 signaling pathway. The presence of the wild-type APC gene in the HT29-APC cells reduced the activity of the c-myc proto-oncogene (He et al. 1998).

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Therefore, it was suggested that the APC tumor supressor gene might modulate the transcription of c-myc proto-oncogene. The APC tumor suppressor gene may directly modulate the transcription of the proto-oncogene c-myc, whereas the mutations of the APC tumor suppressor gene led to the induction and over-expression of the c-myc oncogene expression (He et al. 1998).

In considering this cell model, however, it must be noted that the transfected HT29-APC cells derived from the HT29 human colon cancer cells. The HT29 cells contain APC gene mutations on chromosome 5q21, as well as a mutuated p53 gene and a wild-type ras (Huang et al. 1994). As a result, the induction of the wild-type APC gene in the HT29-APC cells did not eliminate the APC and the p53 gene mutations. The wild-type APC expression presumably corrected the deficit in APC function in the HT29-APC cells. Therefore, this issue was important to consider when interpreting the data in the present study on the role of the presence of the wild-type APC gene in relation to the effectiveness of apigenin to induce cell cycle arrest in colon tumor cells.

It was crucial to determine the time period required for the induction of the wild-type APC gene in the HT29-APC cells, which was manifested by an increase in apoptosis in the $ZnCl_2$-treated HT29-APC cells. We faced the potential problem that the time period required to induce apoptosis in the HT29-APC cells (induction of wild-type APC expression) might occur before the effect of apigenin on cell cycle arrest is observed in these cells, thus affecting the cell cycle arrest data interpretation. Therefore, TUNEL assay was performed to assess the induction of apoptosis in the $ZnCl_2$-treated HT29-APC cells (induction of wild-type APC expression) with incubation time up to 120 hr, in order to conduct the experiments involving treatments with apigenin and/or $ZnCl_2$. The TUNEL assay results shown in

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Chapter two revealed a moderate increase and a more striking increase in apoptosis at 48hr and 120hr, respectively, after treatment with $ZnCl_2$ in the HT29-APC cells. Therefore, this time frame was adequate for our experiments with apigenin and $ZnCl_2$ treatment, as the G2/M cell cycle arrest was observed at 48hr after treatment with apigenin.

Interestingly, the HT29-APC cells revealed a higher percentage of apoptotic cells when treated with 80μM apigenin and $ZnCl_2$ compared to 0μM apigenin and $ZnCl_2$, which was not anticipated in our hypothesis. The HT29-GAL cells, serving as a control for the effect of zinc, showed no changes in apoptosis when treated with zinc. It is also noteworthy that the decrease in HT29-APC cells was more prominent when treated with 80μM apigenin and 100μM $ZnCl_2$ compared to the treatment with 0μM apigenin and 100μM $ZnCl_2$, which paralleled the observed TUNEL assay results. While it appeared that apigenin may have enhanced the sensitivity of the HT29-APC cells to apoptosis when expressing the wild-type APC gene (following treatment of $ZnCl_2$), these data suggested that the treatment of both apigenin and $ZnCl_2$ may have triggered other cellular regulatory pathways.

Because the cell model system applied in this study proved useful to investigate the effect of apigenin in presence or absence of the wild-type APC gene, future studies may involve looking into other similar cell model systems with different inducible genes such as ras and p53. These findings may contribute significantly to the better understanding of the role of dietary factors in relation to the tumors with different mutations. In addition, it may also be speculated that other flavonoids found in plant foods would have a similar inhibitory effect as apigenin on the human colon cancer cells. The normalization of certain cellular functions, which are absent in tumors, would be expected to block the inhibitory effect of the flavonoids. Therefore, patients with certain genetic susceptibilities to colon cancer such as

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FAP (familial adenomatous polyposis) would certainly benefit of the potential chemoprevention suggested through the consumption of a diet rich in fruits and vegetables.

In conclusion, we proved our proposed hypothesis showing that apigenin induced cell growth inhibition by blocking cell cycle progression at G_2/M phase in the HT29-APC and HT29-GAL cells. As expected, the wild-type APC gene expression in the $ZnCl_2$-induced HT29-APC cells was associated with the inability of apigenin to induce a G_2/M cell cycle arrest. The HT29-GAL cells revealed an apigenin-induced G_2/M cell cycle arrest, regardless of the presence of $ZnCl_2$, since they did not express wild-type APC expression. Therefore, our data suggest that apigenin appeared to be more effective in inducing G_2/M cell cycle arrest in tumors with APC mutations. These findings promise to yield new insights into how dietary factors may help prevent colon cancer in patients with certain genetic susceptibility.

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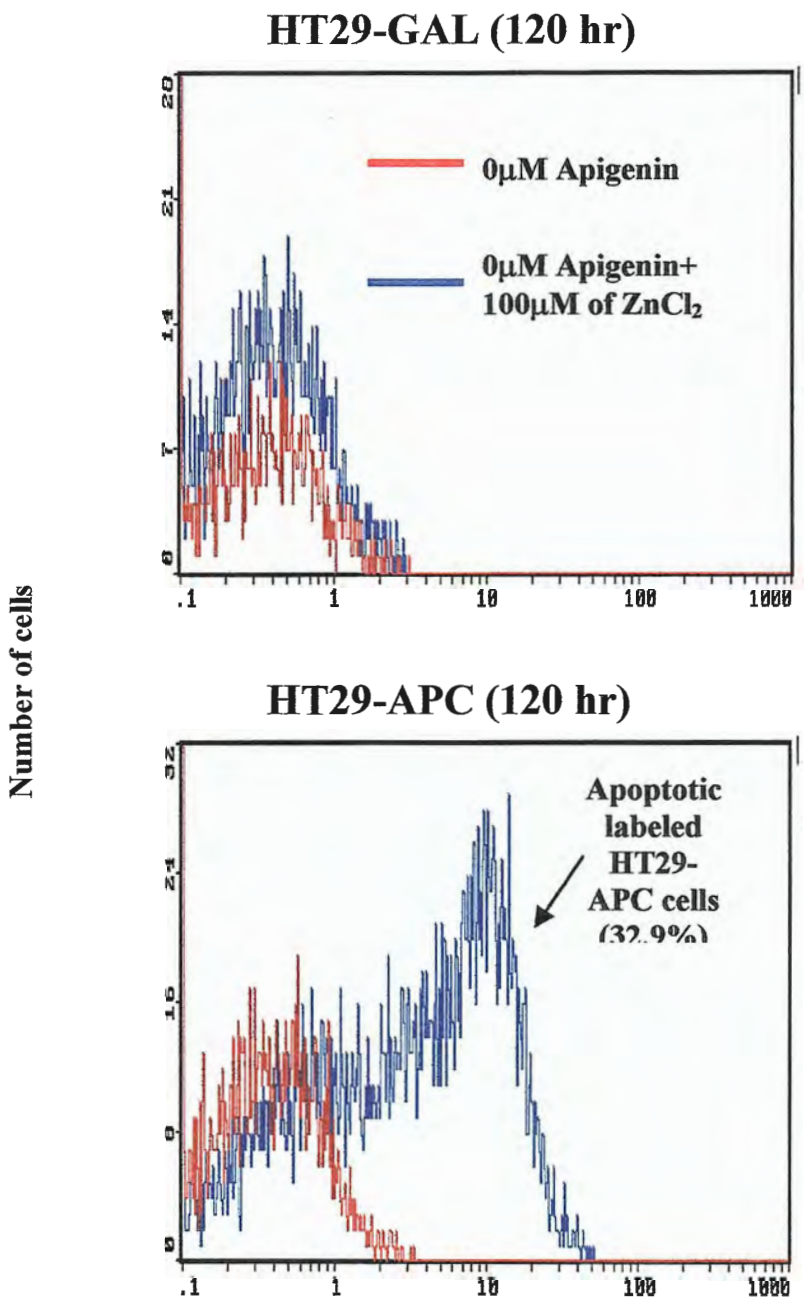

FITC fluorescence intensity (DNA fragmentation presented by BRDU incorporation)

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