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thesis entitled

Tyrosine Phosphorylation Status of Proteins in Rat Colon Mucosa and in the Colon Cancer Cell Lines HT-29 and HCT 116

presented by

Mridvika

has been accepted towards fulfillment
of the requirements for

Masters degree in Human Nutrition

Maurice R. Bennink
Major professor

Date October 17, 1996

TYROSINE PHOSPHORYLATION STATUS OF PROTEINS IN RAT COLON MUCOSA AND IN THE COLON CANCER CELL LINES HT-29 AND HCT 116

By

Mridvika

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1996

ABSTRACT

TYROSINE PHOSPHORYLATION STATUS OF PROTEINS IN RAT COLON MUCOSA AND IN THE COLON CANCER CELL LINES HT-29 AND HCT 116

BY

MRIDVIKA

A study was conducted to determine protein tyrosine phosphorylation status in colon mucosal cells of rats treated with 1,2-dimethylhydrazine and in HT-29 and HCT 116 colon cancer cells at three points in the cell cycle. Synchronized colon cancer cells were harvested at 0, 0.5, and 8 hrs after being induced to enter the cell cycle. Extracted proteins were resolved with 2-dimensional electrophoresis and tyrosine phosphorylated proteins were detected using specific antibodies. In HT-29 colon cancer cells four proteins (MW's 50, 56, 56 and 77 KD) had changed tyrosine phosphorylation status at different points in the cell cycle and in HCT 116 cells, ten such proteins (MW's 28 to 92KD) were detected. Two proteins of MWs 50 and 57KD were common to both cell lines. In rat colon mucosa seven proteins in molecular weight range 36-67 KD were found to be tyrosine phosphorylated to a greater or lesser extent on the third and seventh day after DMH treatment.

To my grandmother, mother and sister; three generations of influential women whose mentoring I enjoy.

ACKNOWLEDGMENTS

I would like to sincerely thank my major professor, Dr. Maurice Bennink for his guidance, understanding, moral support and constant encouragement. Thanks are also extended to members of my committee, Dr. Dale Romsos and Dr. William Helferich for their guidance and patience throughout the program.

I would like to thank all my colleagues, and especially Dr. Leslie Bourquin for his valuable input in this project. Many thanks to my friends who have always been there for me.

Finally, I would also like to extend heartfelt thanks to my dear family whose love and support have always been my inspiration to achieve and excel.

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INTRODUCTION

Colon cancer claims the lives of nearly 51,000 people in the United States every year. The incidence of colon cancer is related to the consumption of a western type diet which is high in fat and low in fiber. A number of studies have reported positive correlations between consumption of fat (Stemmerman, Nomura and Heilbrun, 1984), meat or animal protein (Enstrom, 1975) and colon cancer incidence (Thun, 1992; Howe, 1992) strongly suggesting a link between diet and colon cancer.

Genetically, colon cancer is the combined result of several mutations in the cells of the colon mucosa. These mutations can result in either activation of oncogenes or inactivation of tumor suppressor genes (Fearon and Vogelstein, 1990). It has been suggested that the chronological order in which these mutations come into effect is not relevant to the assessment of the risk for developing colon cancer. The severity of the risk for colon cancer is related to the number of accumulated mutations (Fearon and Jones, 1992). People with one or two mutations

might have a normal appearing mucosa and still be at risk for developing the disease. It is this group of people that are likely to benefit most from dietary intervention of some kind. To assess the risk for colon cancer in a clinical setting, bio-markers have been developed.

Studies have shown that hyperproliferation of the normal appearing colon is probably the earliest detectable abnormality observed in people pre-disposed to colon cancer (Lipkin, 1974; Deschner, 1978). Hyperproliferation is the condition in which the dividing cells of the colon fail to differentiate as they normally would into mature cells and remain in a proliferating mode and is considered an early bio-marker for colon cancer, measured by radio-labeling or immuno-labeling in histological sections. These techniques are time and labor intensive and require specific technical training. Therefore, a protein marker for hyperproliferation would offer an advantage in detection of colon cancer risk.

The objective of my study was to identify proteins where tyrosine phosphorylation reflected the proliferation state of the colon mucosal cells. This was studied in HT-29 and HCT 116 colon cancer cells and 1,2-dimethylhydrazine treated animals. The proteins were studied using two dimensional electrophoresis and immuno-detection with monoclonal antibodies.

REVIEW OF LITERATURE

A. EPIDEMIOLOGICAL REVIEW

A.1. Incidence of Colon Cancer

The past hundred years have seen a marked decline in deaths caused by infectious diseases and an increase in expected life span. As a result, cancer and cardiovascular disease, traditionally "diseases of the old age" incidence continues to increase in a population that is rapidly aging. Whereas, the downward trends in cardiovascular disease have been encouraging, after decades of intensive research, cancer still continues to be a major cause of mortality in the US with colon cancer being among the deadliest of cancers. A 1996 publication of the American Cancer Society reported 133,500 new cases in 1996 of colon cancer and rectum cancer combined, making it the fourth most incident cancer (after breast, lung and prostrate) and the second most common cause of cancer death (after lung) in the US

(Cancer Facts and Figures, 1996).

A.2. Distribution of Colon Cancer

Epidemiological studies have shown that colon cancer incidence varies about twenty-fold across countries of the world. The first indication of such a variation came from Segi's compilations of cancer mortality statistics for the years 1950-1957 (Segi, 1960). At the time, it was suspected that these intercountry differences were inflated as a result of differences in diagnostic and treatment facilities and death certification practices across countries. Subsequent studies have refuted these doubts and confirmed Segi's findings (Muir et al., 1987; Parkin et al., 1992). The highest rates of CC incidence are found in the developed world - North America, Western Europe and Australia- with age adjusted incidence rates of 25-35 cases per 100,000 in the late 1980's. In contrast, much lower rates occur in countries of Asia, Africa and southern Europe with the lowest rates found in India, that is, 1-3 per 100,000 people (Cancer Facts and Figures, 1992).

A.3. Diet and Colon Cancer

Following the observation of the disparate rates of colon cancer, differences in diet patterns across countries

were suggested as a possible explanation. Burkitt hypothesized that a low-bulk, high starch diet might be involved in the causation of colon cancer (Burkitt, 1969). Since then, many epidemiological studies have examined correlations between the consumption of certain nutrients and non-nutrients and the incidence of CC. The focus of most of these studies has been fat, meat, animal protein, fiber or cereal intakes. A number of studies have reported positive correlations between consumption of fat (Stemmerman, Nomura and Heilbrun, 1984), meat or animal protein (Enstrom, 1975) and colon cancer incidence (Thun, 1992; Howe, 1992). Some research has examined the possibility that the high energy intake associated with such diets might be the risk factor for colon cancer incidence (Lyon et al., 1987). Extensive work also has been conducted to ascertain the link between lifestyle patterns and colon cancer incidence. Seventh-Day Adventists abstain from smoking and drinking and most of them consume an ovo-lacto-vegetarian diet, a diet including eggs and milk but excluding meat. A reduced risk for colon cancer has been reported in this population relative to the rest of the population (Phillips and Snowdon, 1985; Phillips, 1975). A lower colon cancer risk also has been associated with consumption of diets with a high fiber content (Burkitt, 1969 ;Howe, 1992). In most of these studies, comparisons of

incidence and mortality from colorectal cancer and diet across populations are based on food disappearance data obtained from the Food and Agricultural Organization (FAO). All these studies indicate that diet plays a significant role in colon cancer.

The incrimination of environmental factors rather than genetic factors as important risk determinants for colon cancer was further corroborated by studies of migrant populations. It was observed that migrants moving from a low risk area to a high risk area acquired, within their lifetime, the high risk characteristic of the host population. For example, Japanese migrating to the US exhibit the high risk rates for colon cancer typical of US whites rather than the low risk rates typical of Japan (Haenzel and Kurihara, 1968).

B. CELL PROLIFERATION AND DIFFERENTIATION IN COLON CANCER

The high mortality rates due to colon cancer have led to enormous interest in early detection and treatment of the disease. Also, since the disease has a major dietary component, detection of predisposition to colon cancer can make dietary intervention possible at a stage early enough to make a difference. To make early detection possible, the proliferation and differentiation characteristics of colon

mucosal cells have been studied extensively. Many studies have shown that epithelial cell proliferation and differentiation is modified in conditions predisposing to colon cancer.

Normal colon cells are columnar, mucous secreting, or enteroendocrine and are arranged in crypts. In a normal colonic crypt most of the DNA synthesis activity is confined to the lower one-third of the crypt. Cell proliferation is almost always completed in the lower two-thirds of the crypt (Lipkin, 1988). The region of the crypt where cell division occurs is termed the proliferative zone. The dividing cells at the bottom of the crypt differentiate and mature as they migrate up the crypt toward the luminal surface. By the time these cells reach the luminal surface they are mature, differentiated, non-proliferating cells (Maskens and Deschner, 1977). Rarely does cell division occur at the luminal surface of normal mucosa. This scenario is radically altered in certain diseased states. An expansion of the proliferative zone (also referred to as hyper-proliferation), expansion of the major zone of DNA synthesis, and high levels of DNA synthesis within colonic crypts are some of the changes observed in the mucosa of patients with gastrointestinal disturbances (Lipkin and Higgins, 1988). Hyper-proliferation usually means an increase in the mitotic index, which is a ratio of the

number of dividing cells in a crypt to the total number of cells in the crypt.

Studies of proliferation and differentiation of colonic mucosal cells have helped identify the stages of abnormal development associated with an increased susceptibility to colon cancer (Deschner, 1978; Lipkin, Venara and Winawer, 1985). Studies of normal appearing colon mucosa of people at greater risk for developing colon cancer have shown that hyperproliferation of the colonic mucosa is among the first abnormalities detected. This hyperproliferation has been observed in subjects with familial polyposis, sporadic adenomas, or previous histories of familial or non-familial colon cancers (Lipkin, 1974). Hyperproliferation leading toward an expansion of the proliferative zone toward the luminal surface corresponds to stage 1 in the development of adenomas. In stage 1, the major zone of DNA synthesis, however, continues to be the lower one-third of colonic crypts. This stage has been observed in the colonic mucosa of people with familial polyposis, isolated adenomas, colon cancer and relatives of individuals who have familial polyposis (Deschner and Lipkin, 1975). The next stage in adenoma formation, stage 2, is characterized by a shift in the principal zone of DNA synthesis from the lower one-third toward the middle and upper thirds of the crypt (Maskens and Deschner, 1977). This stage was first identified in the

colonic mucosa of people with a previous history of colon cancer and was later observed in other high risk groups, such as, familial polyposis, non-symptomatic relatives of polyposis patients, people with sporadic polyps and colon cancer patients and has never been observed in people with no history of gastrointestinal disease (Deschner, 1980). Stage 3 of adenoma development has been described as the presence of isolated crypts with high levels of DNA synthesis activity (Deschner and Maskens, 1981). The labeling index (LI, the percentage of dividing cells in a crypt) of these crypts was reported to be greater than 15%. Only one out of 13 control patients (no gastro-intestinal abnormality) had such crypts, but 17 out of 26 patients with a history of colon cancer or isolated adenomas were positive for the presence of these hyper-proliferative crypts.

Hyper-proliferation and an upward shift in the proliferative zone result in appearance of mitotic cells at the mouth of the colonic crypts and in the flat mucosa. It is believed that this leads to an accumulation of cells near the mouth of the crypt in an otherwise flat mucosa. The accumulated cells eventually form polyps which initially are small and benign. With further changes over a period of time, some of these polyps grow and develop into adenomas.

It has been shown that most, if not all carcinomas are formed from pre-existing adenomas.

Abnormal cell proliferation and differentiation and pre-neoplastic gene expression are considered to be biomarkers of an increased risk of colon cancer (Lipkin, 1988). Measurements of cell proliferation and differentiation also aid research concerned with cancer prevention through dietary intervention. These measurements have aided the development and application of intermediate biomarkers to studies of cancer prevention, thus precluding the use of actual neoplasia as the endpoint.

C. EXPERIMENTAL MODELS IN CANCER RESEARCH

C.1. Animal models

The major advantage afforded by the use of animals in cancer research is that it allows researchers to study the various factors in diet which might individually play a role in cancer promotion or prevention. Most of the animal models are based on the administration of certain chemicals either orally, subcutaneously or intravenously. These chemicals cause mutations leading to increased cell division in target tissues that results in the formation of tumors which are

benign to begin with, and ultimately become cancerous as additional mutations occur.

The use of natural cycasin or synthetic chemicals to induce colon cancer in rats has been utilized for many years (Laqueur and Spatz, 1968). Currently, the most commonly used chemicals for inducing colon cancer in the rat are 1,2-dimethylhydrazine (DMH), methylazoxy-methanol (MAM) or MAM acetate (MAMAc). Since the introduction of symmetrical DMH as a specific carcinogen for the colon by Druckery and his associates, it has been used widely for investigating colon carcinogenesis (Druckery et al., 1975). DMH induces hyper-proliferation and other manifestations of abnormal cell division and differentiation found in human colon mucosa progressing towards malignancy. It is postulated to do so by inducing aberrations in the normal sequence of DNA replication and repair. In addition, it is also extremely cytotoxic, so 1-3 days after administration, hyperproliferation occurs to replace the dead cells. The detoxifying enzymes in the liver convert DMH into the activated methylating agent, methyl-azoxy-methanol and the oxidative demethylation of the activated MAM results in the methylation of the purine base guanine in both RNA and DNA. Guanine methylation results in the introduction of changes in the DNA sequence (mutations) during replication. The introduction of these changes in a rapidly proliferating

tissue such as the colonic epithelium leads to the formation of tumors over a period of time.

In a number of studies Deschner and co-workers have demonstrated the proliferative defects induced by 1,2-dimethylhydrazine. In 1973, Thurnherr and Deschner reported an increased number of proliferating cells close to the luminal surface of colonic mucosa of mice in response to seven weekly injections of DMH when compared to controls which did not receive any DMH, even though the histology was normal and the labeling index was unchanged (Thurnherr et al., 1973). After 87 days, the colonic mucosal labeling index of DMH-treated mice was 16.2%, whereas control mice had a labeling index of 6.2%. At this time, the morphology of colonic crypts from DMH-treated mice was also deranged. In a similar study (Deschner, 1978), histological changes in mice were observed in response to six weekly injections of DMH. Labeling index peaked on the second and third days after carcinogen administration to about 19% (compared to 6.6-10.7% for untreated controls), dropped to values close to those of the controls, but rose again at week 7 after carcinogen administration. In 1982, Deschner and Maskens reported colonic mucosal cell proliferation in a group of cancer patients and in DMH-treated mice (Deschner and Maskens, 1982). The colonic mucosa of patients with sporadic polyps was characterized by the presence of isolated crypts

with labeling index greater than 20%; such crypts were absent in control subjects that had no gastrointestinal disease. The distribution of proliferating cells in crypts with labeling index greater than 15% was compared to crypts with labeling index between 6-8%. Crypts having both high and low labeling index demonstrated the Stage 1 and Stage 2 abnormalities described previously, but they were more emphatically expressed in crypts with labeling index greater than 15% (Deschner and Maskens, 1982). Isolated crypts of DMH-treated mice had labeling indices between 21 to 38%, whereas labeling index in crypts from untreated control mice never approached 21%. In the control mice, proliferating cells were located exclusively in the lower two-thirds of colonic crypts. Crypts of the DMH treated mice showed an extension of the proliferative compartment closer to the lumen and an upward shift in the major zone of DNA synthesis (Deschner and Maskens, 1982).

C.2. Cell Culture and Cancer Research

Animal models have contributed a great deal to our understanding of colon cancer and the influence of nutrition on cancer development, but these studies are often limited by their long duration and high cost. In recent years several colon cancer cell lines derived from human tumors

become commercially available. Since it is possible to control for factors that can influence tumor development in cell culture, these techniques have made a large contribution to our understanding of colon cancer. Much attention has been focused on the development of dependable, early neoplastic markers in cell culture. Mutations which initialize early steps in cancer cause changes in proteins. There is potential to tap into one of these changes and use as a marker.

D. GENETIC BASIS OF COLON CANCER

D.1. Mutations in Colon Cancer

The changes in the proliferation of cells of the colon mucosa are caused by alterations in cell cycle control such as delayed onset of normal terminal differentiation. Colon cancer appears to be the cumulative result of mutational activation of oncogenes and inactivation of tumor suppressor genes (Fearon and Vogelstein, 1990). Some of the molecular events underlying the initiation and progression of the multistep process of tumorigenesis described have been identified (Fearon and Vogelstein, 1990). Three stages of adenoma development have been described. Early stage adenomas are defined as adenomas less than one cm in size. Intermediate stage adenomas are greater than one cm in size,

but do not contain foci of carcinoma. Late stage adenomas are greater than one cm in size and contain foci of carcinoma (Fearon and Jones, 1992). The genetic changes associated with the progression of an early adenoma to carcinoma include mutations of the K-ras gene, loss of heterozygosity (LOH) at certain loci, and allelic deletions in chromosomes 17p and 18q (Fearon et al., 1990). The gene deleted in chromosome 18q, called DCC (Deleted in Colon Cancer), is missing in 50% of late adenomas and 70% of carcinomas. This gene encodes for a protein with significant homology to the cell adhesion family of molecules. Absence of the protein product of this gene probably alters cell adhesion, causing diminution of growth restraining signals associated with such adhesion. The 17p protein product, p53, is a tumor suppressor protein whose deletion often marks the conversion of adenomas into carcinomas (Fearon and Jones, 1992).

A genetic model for the development of colon cancer proposed by Fearon and Jones in 1992 relates the timing of the genetic events to the development of colon cancer (Fearon and Jones, 1992). People with familial polyposis have a germ-line mutation in the APC gene, that results in hyperproliferation of the colonic epithelium. Somatic APC (adenomatous polyposis coli) gene mutations (chromosome 5q LOH) can result in similar changes in patients without

familial polyposis. Hyperproliferation of the colonic epithelium precedes the formation of adenomas. The transformation of early adenomas to intermediate adenomas is often associated with K-ras gene mutation. Additional events such as chromosome 18q LOH (the DCC mutation), and chromosome 17p LOH (p53 mutation) often mark the transition of a late adenoma to a carcinoma.

D.2. Alternative Modes of Regulation of Cell Number

The underlying cause of cancer is often thought to be the loss of control on cell proliferation. Recently this idea has undergone a change. With the realization that cell number is a result of opposing effects of cell proliferation and cell death, the latter has taken on greater significance. Since recent studies have suggested that cell death, like cell proliferation, is subject to molecular control, thus cancer can result from loss of regulation of either cell proliferation or cell death or the combination of two (Green et al., 1996).

Apoptosis, or programmed cell death, is of particular importance in this regard. One of the observations that support this claim is that most physical and chemical agents that have antitumor activity act to induce apoptosis (Lennon, Martin and Cotter, 1991; Martin and Green, 1994;

Martin and Green, 1995). In addition, it has been noted that frequency of apoptotic cells in tumor correlates very well with the outcome. That is, tumors with low apoptotic indices tend to be more aggressive than those displaying higher incidence of apoptotic cell death (Arends, McGregor and Wyllie, 1994). The observation that links apoptosis to protein phosphorylation is that the process of apoptosis is regulated by a number of oncogenes and anti-tumor oncogenes (Martin and Green, 1994; Martin and Green, 1995; Bissonnette et al., 1994), many of which are found to be mutated en route to cancer.

The proteins involved in apoptosis can be grouped into two categories - the ones that promote apoptosis and the ones that inhibit apoptosis. Interestingly the effects of these two classes of proteins on transformation are opposing to their effect on apoptosis, which is consistent with expected relationships between these activities. Bcl-2 (Korsemeyer, 1992; Reed, 1994) and Abl (McGohan, Cotter and Green, 1994) promote transformation and block apoptosis, while p53 acts to interfere with oncogenesis and promotes some forms of apoptosis (Yonish-Rouach et al., 1991; Shaw et al., 1992; Lowe et al., 1993). In view of these observations, the activities of Myc and Ras are perhaps surprising. Ras and Myc are two proto-oncogenes that

function in normal cells to promote proliferation but are found to promote apoptosis under some conditions (Bissonnette et al., 1994; Wyllie et al., 1987; Askew et al., 1991; Evan et al., 1992; Tanaka et al., 1994).

The Bcl-2 family of proteins are a family of unique proteins. The protein encoded by the BCL-2 gene, for example, bears no significant amino acid homology with other proteins whose biochemical mechanism of action is known. From the analysis of the amino acid sequence, it appears that Bcl-2 is an integral membrane protein whose transmembrane domain and C terminal region are essential for optimal function (Cazals-Hatem et al., 1992; Krajewski et al., 1993; Tanaka et al., 1993; Borner et al., 1994). At present, six mammalian homologs of Bcl-2 have been reported, including Bax, Bcl-X, Mcl-1, A1, Bad and Bak (Oltvai et al., 1993; Boise et al., 1993; Kozopas et al., 1993; Lin et al., 1993a; Yang et al., 1995; Chittenden et al., 1995; Kiefer et al., 1995 ; Farrow et al., 1995). Of these, the Bax, Bad and Bak proteins function as promoters of cell death. Conversely, the Mcl-1 and A1 proteins appear to be suppressors of cell death, along with Bcl-2.

Although the BCL-2 gene was first discovered because of it's role in t(14;18) translocations found in non-Hodgkin's lymphoma, high levels of BCL-2 gene expression have been found in a variety of human cancers including 90% of

colorectal cancers (Reed, 1994; Reed, 1995). Moreover, BCL-2 gene expression has been reported to be suppressed by p53, through direct or indirect mechanisms. In experiments where p53 function was conditionally restored to a p53-deficient murine leukemia line, p53 was shown to induce marked decreases in bcl-2 gene expression followed by apoptotic cell death (Miyashata et al., 1994). The p53 gene located on chromosome 17p is mutated in greater than 70% of human colorectal cancers.

E. PROTEIN TYROSINE PHOSPHORYLATION

E.1. Tyrosine Kinases and Cancer

Phosphorylation and dephosphorylation of cellular proteins at their tyrosine, serine or threonine residues is a predominant method for regulating enzyme activity. Many of these proteins have a role in regulating the cell cycle. Protein tyrosine kinases (PTKs), therefore, were an obvious choice for researchers in their quest to better understand the molecular mechanisms underlining the cellular changes associated with cancer (Hunter and Cooper, 1985).

The highly conserved sequences of the genes encoding PTKs in species ranging from humans to simple eukaryotes suggest the importance of tyrosine phosphorylation in cellular physiology (Hunter and Cooper, 1985). Today we

recognize two major classes of PTKs - (I) those whose genes have become a part of retroviruses, and (ii) those associated with growth factor receptors.

The genes for the type of PTK's that become a part of the retroviruses are present in the normal eukaryotic genome as proto-oncogenes. The protein products of these proto-oncogenes are membrane PTK's. These proteins have an exoplasmic ligand binding site, a trans-membrane part and a cytoplasmic kinase site. Under normal conditions, the binding of a specific ligand on the ligand-binding site of the PTK activates the kinase to phosphorylate cellular proteins. This can trigger a cascade of events that culminates in a response by the cell, cell division being one such response. The capture of receptor tyrosine kinases (RTK) into the genomes of acutely transforming retroviruses has been one of the predominant mechanisms by which these molecules become overexpressed or inappropriately expressed. For example, the RTK's *erb*B (chicken) (Ulrich et al., 1984), *sea* (chicken) (Smith et al., 1989), *kit* (feline) (Besmer et al., 1986), and *fms* (feline) (Hampe et al., 1982) were first uncovered in this fashion. Both overexpression and mutation appear to be features of the RTK sequences captured by retroviruses. N-terminal truncations usually remove a significant portion of the extracellular ligand binding

domain and are found in v-*kit* (Besmer et al., 1986), v-*erb*B (Ulrich et al., 1984) and v-*sea* (Smith et al., 1989). Thus, a mutation resulting from the action of a carcinogen can lead to the conversion of a proto-oncogene to an oncogene that codes for a PTK with the exoplasmic ligand binding site either missing or dysfunctional. The activation of the kinase activity of this protein thus becomes independent of ligand binding (Hunter and Cooper, 1985). Such a cell receives signal to divide even in the absence of external stimulus, throwing the cell into a hyper-proliferative state.

A good example of this would be the overexpression or aberrant expression of epidermal growth factor(EGF) receptors that have been reported in cell lines derived from gastric cancer (Yasui et al, 1988) and squamous cancer of various origins such as skin, tongue, esophagus, gingiva and vulva (Yamamoto et al, 1986). On the other hand, activated $pp60^{c\text{-}src,}$,a known cytoplasmic kinase, has been observed to be activated in colon carcinoma cells (Cartright, Meisler and Eckhart, 1990).

E.2. Cell Cycle and Cancer

Accumulating evidence suggests that derangements in the cell cycle machinery may contribute to the uncontrolled cell

growth characteristic of a tumor. Over the past few years, cell biologists have made remarkable progress in understanding of the control of the cell cycle and identification of the proteins and factors that drive it.

The eukaryotic cell cycle consists of a phase of chromosomal DNA replication (S phase) followed by segregation of the replicated chromosomes into two daughter cells during M phase. In most animal cells, gap phases G1 and G2 occur between M and S phases and between S and M phases, respectively. During these gap phases, information is integrated by the cell to determine the readiness of the cell to enter either S or M phase. Thus, the G1/S and G2/M boundaries serve as major checkpoints for control of progression of the cell through the cell cycle (Norbury and Nurse, 1992).

Research on cell cycle control in several distinct experimental systems has recently converged. Today, much is understood about the proteins comprising the machinery which drives the eukaryotic cell cycle. The key regulators of cell cycle progression are proteins of the cdc2 protein kinase family ($p34^{cdc2}$), a highly conserved family of proteins in eukaryotic cells. The kinase activity of these proteins is dependent upon the association with another highly conserved class of proteins called cyclins. Thus the $p34^{cdc2}$ family of proteins are often referred to as cdks (cyclin-dependent

kinases) (Motokura and Arnold, 1992). Cyclins fluctuate in level during cell cycle progression. Individual cyclins typically peak in concentration during the stage of cell cycle in which they are required and are degraded once that point has passed (Doree and Galas, 1994).

E.3. Cell Cycle and Tyrosine Kinases

Different combinations of cdks and cyclin subunits form kinase complexes which act at different points in the cell cycle (Norbury and Nurse, 1992). In addition to association with the different cyclin subunits at different stages of the cell cycle, cdk activity is regulated by a multiple phosphorylation events also. The best characterized example is an inhibitory tyrosine phosphorylation Tyr-15 of $p34^{cdc2}$ in the ATP-binding domain. The state of tyrosine phosphorylation of $p34^{cdc2}$, and thus the entry into mitosis, is determined by a balance of kinase and phosphatase activities. A family of protein kinases, $p107^{wee1}$ phosphorylate Tyr-15 of $p34^{cdk}$'s inhibiting it's kinase activity, thereby preventing the onset of mitosis (Russel and Nurse, 1987). A family of $p34^{cdc2}$ specific tyrosine phosphatases ($p80^{cdc25}$) dephosphorylate the same site, activating $p34^{cdc2}$ and permitting entry into M phase.

Phosphorylation has also been implicated in regulating

the abundance of G_1 cyclins in the cell. In yeast phosphorylation of Cln 2, a G_1 cyclin that binds Cdc 28 provides the signal that is necessary for it's rapid degradation. It is speculated that Cdc 28 is the primary kinase that phosphorylates Cln 2. Targeting Cln 2 for degradation by means of phosphorylation it's cognate CDK provides a mechanism by which activation of the Cdc 28 CDK by Cln 2 could target that cyclin for degradation, thus rendering it self-limiting (Lanker et al., 1996).

Thus, the regulation of cell entry into mitosis can be affected by the balance of competing kinases and phosphatases. Each of these protein families are further regulated by a multitude of phosphorylation events. The activity state of any of these crucial proteins can be affected by upstream events triggered by extracellular signals. Many extracellular signaling pathways converge on a protein kinase family termed the mitogen-activated protein (MAP) kinases (Pelech et al., 1993). After stimulation, MAP kinases phosphorylate a broad spectrum of substrates involved in different cellular functions and events reflecting the central position of these protein kinases in the cellular phosphorylation network (Mordet, 1993). Activation of the MAP kinase pathway regulates translation through *rsk* (Blenis, 1993) and protamine kinase (Reddy et

al., 1993) which phosphorylate eukaryotic protein synthesis initiation factor eIF4-E. MAP kinases also regulate transcription by phosphorylating nuclear transcription factors (c-jun, c-myc, c-tall, etc)(Cheng et al., 1993; Pulverer et al., 1991; Pulverer et al., 1993; Seth et al., 1991). The activities of MAP kinases also include the regulation of microtubule dynamics (Gotoh et al., 1991), metabolism (Merral et al., 1993; Pelech et al., 1991), and release of second messengers such as arachidonic acid (Lin et al., 1993). MAP kinases are also postulated to be involved in cell cycle control and tumor proliferation though the interaction between MAP kinases and the cdk/cyclin complexes are not yet fully understood (Ahn, 1993).

E.4. Protein Tyrosine Kinases in Colon Cancer

PTK activity in human colon carcinoma has been found to be altered in comparison to surrounding normal tissue (Sakanoue et al., 1991). Examination of PTK activity in cytosolic and particulate fractions of homogenates from twenty human colon carcinomas revealed an increase in the PTK activity in the particulate fraction and decrease in the PTK activity in the cytosolic fraction when compared to normal adjacent tissue (Sakanoue et al., 1991). In addition, a marked

increase in tyrosine kinase activity was observed to accompany a rise in ornithine decarboxylase activity in rectal mucosa from patients with adenomatous or hyperplastic polyps (Colarian et al., 1991). A similar phenomenon was observed in the colonic mucosa of rats in response to the carcinogen azoxymethane (AOM), that is, an increase in the tyrosine kinase activity was observed as the ornithine decarboxylase activity increased (Arlow et al., 1989). In another study maximal increase in the tyrosine kinase was reported to occur five days after the AOM administration and was 70% over the control. This rise in tyrosine activity could be partially attributed to increased tyrosine kinase activity of the epidermal growth factor receptor (EGF-R). Interestingly, the increase in the tyrosine kinase activity of the EGF-R is caused by a 250% increase in the tyrosine phosphorylation of the receptor itself (Relan et al., 1995). The exposure of human colon cancer cell lines to differentiation promoting factors like sodium butyrate significantly attenuated tyrosine protein kinase activity. This increase concomitant with an increase in the activity alkaline phosphatase, an enzymatic marker of intestinal differentiation indicating that the induction of differentiated phenotype in colon cancer is associated with a marked decrease in tyrosine protein kinase activity and tyrosine phosphorylation (Schwartz et al., 1995).

F. JUSTIFICATION

To briefly summarize the preceding review, colon cancer is accompanied by marked changes in the proliferation state of the colon mucosa. Most of the information about the proliferation in colon mucosa has been provided by histological studies. At present labeling indices and proliferation patterns are the most dependable markers available for assessment of colon cancer risk. Unfortunately, the determination of these assessment markers is a time and labor intensive process. It usually involves multiple steps, namely, fixing, embedding and cutting sections of the tissue followed by a staining process involving either a radioactive labeling technique or immunodetection. The justification for the search of a protein marker is that it is far more convenient and accessible to test for a protein in a clinical or a diagnostic setting. A technique that would enable us to detect changes in proliferation state without doing histology would be of tremendous advantage in diagnosis and early detection. A detection technique that is easier and faster to conduct would make possible for more people to undergo the screening process. If the detection of colon cancer is early, the intervention has a much higher probability of being successful.

G. OBJECTIVE

My objective was to determine if cellular proteins change in detectable levels in relation to the proliferative state of the tissue. Furthermore, in view of the evidence for the role of tyrosine phosphorylation in cell division, changes in protein tyrosine phosphorylation might be expected to reflect changes in proliferation state of the colon mucosa. Following this, my second objective was to detect changes, if any, in tyrosine phosphorylation of proteins that occur in a hyperproliferative state. Serine and threonine phosphorylated proteins, which are also crucial in cellular proliferation, were not a part of this study, being too wide a field to fit the scope of my study.

The experimental approach undertaken to achieve these objectives was to first measure changes in cellular proteins in colon mucosa of rats treated with dimethylhydrazine. This was followed by measuring changes in protein tyrosine phosphorylation. Since the colon mucosa have cells in various stages of the cell cycle at any given time, changes in protein tyrosine phosphorylation, that are very subtle to begin with, might have been difficult to detect. Therefore, changes in protein tyrosine phosphorylation in response to different phases of the cell cycle were examined in a cell culture system where it is possible to synchronize the cells to enter

the cell cycle simultaneously. After development of the technique in cell culture, changes in protein tyrosine phosphorylation in rat colon mucosa were determined.

H. NULL HYPOTHESIS

The null hypothesis to be tested in this study is that the amount of tyrosine phosphorylation in colon mucosal proteins is the same in DMH treated and non-DMH treated rats.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Study 1. Study 1 examined changes in colon mucosa proteins following a single dose of DMH. The study used four rats, two received a single dose of DMH (the dosage of DMH in all the rat studies was 20mg/kg body weight) and the other two rats were given saline with no DMH (controls). All four rats were sacrificed two days following the treatment and the colon mucosal proteins were extracted.

Study 2. Study 2 was conducted to determine changes in colon mucosal proteins three and five days after DMH administration. This study utilized five Sprague-Dawley rats; four were given a single dose of DMH and the fifth rat was given saline. The control and two DMH treated rats were sacrificed on the third day after treatment and the other two rats were sacrificed on fifth day after the treatment. The mucosal proteins were extracted and analysed.

Study 3. A cell culture study was conducted to determine changes in protein tyrosine phosphorylation by two dimensional electrophoresis (2-DE). Cells were induced to cycle

simultaneously and were harvested at times 0, 0.5 and 8 hours after the stimulus had been provided. The cell lysates were analysed for protein tyrosine phosphorylation using 2-DE and western blotting.

Study 4. Ten rats which were randomly assigned to one of three treatment groups: 1) Saline administration with no DMH to be sacrificed on the first day after injections, this group had four rats 2) single dose of DMH and sacrificed om the third day after the injections, this group had three rats and, 3) a single dose of DMH and sacrificed on seventh day after the injections, this group had three rats. The colon mucosal protein were extracted from all of these rats.

ANIMALS AND HOUSING

Seven to eight week old Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The rats were housed individually in stainless steel hanging wire cages. The animal room was temperature (68°-75°F) and humidity (40-70%) controlled and a twelve hour light-dark cycle was maintained. The rats were fed a modified AIN-93(G) diet (Table 1.). All the protocols for animal housing and injections were approved by the All University Committee on Animal Use and Care.

Table 1. Dietary composition of the modified AIN-94 diet fed to rats in studies 1,2 and 4. These quantities are for 10 Kgs of diet.

Ingredients	Quantity (Kg)
Corn Starch	3.49
Casein	2.0
Sucrose	1.0
Corn Oil	1.5
Fiber	0.5
Mineral Mix (AIN-93)	0.35
Vitamin Mix (AIN-93)	0.1
L-Cystiene	0.03
Choline Chloride	0.025

CHEMICALS AND REAGENTS

Dietary components were obtained from Teklad Test Diets (Madison, WI). Sym-1,2-dimethyl-hydrazine was purchased from Sigma Chemical Company (St. Louis, MO). The polyvinylidene diflouride (PVDF) membrane and the Rennaissance Chemiluminescence used for the detection of proteins were obtained from DuPont NEN Research Products (Boston, MA). Anti-phosphotyrosine-horseradish peroxidase conjugate, goat anti-mouse IgG-horseradish peroxidase conjugate and anti-PCNA monoclonal antibody were all purchased from Oncogene Science (Uniondale, NY). Bicinchoninic acid was obtained from Pierce Chemical Company (Rockford, IL). Cell lines HT-29 and HCT 116 were purchased from American Type Cell Culture Association (Bethesda, MD). Fetal bovine serum was obtained from Life Technologies (Giethersburg, MD). Penicillin/streptomycin stock and all other chemicals were obtained from Sigma Chemical Company.

METHODS

Dimethylhydrazine Administration to Rats: Colonic epithelial cell proliferation was induced by a single subcutaneous injection of DMH. DMH was administered in a 0.15M saline, 1 mM EDTA solution with the concentration adjusted such that 0.1 ml contained 20 mg DMH/ kg body weight. The pH

of the solution was adjusted to 7.4 with sodium carbonate prior to injecting the animals.

Tissue Collection and Preparation: Rats were sacrificed by carbon dioxide asphyxiation followed by exsanguination. The entire colon was removed, cut open longitudinally, and rinsed briefly with cold running water followed by PBS (ice-cold) to remove fecal debris. Mucosal cells were scraped off with a glass microscope slide and homogenized immediately in homogenization buffer [20mM 3-[N-morpholino]propanesulphonic acid, 150mM NaCl, 1% triton X-100 (v/v), 1% deoxycholate(v/v), 0.1% sodium dodecyl sulphate(v/v), 1mM ethylenediamine-tetraacetic acid.Na_2, 100µM sodium orthovanadate, 250µM phenylmethylsulphonyl flouride, 10µg/ml leupeptin, 1µg/ml pepstatin] for studies 1 and 2, and lysis buffer [9.5 M urea, 2% nonidet P-40 detergent, 2% ampholytes(pH 3-10), 5% β-mercaptoethanol] for study 4. Since the ampholytes and the β-mercapto-ethanol can interfere with the protein estimation, these were added after protein estimation. The homogenates were centrifuged at 14,000 rpm for ten minutes to obtain a clear protein extract which was used for 2-DE. The protein content of homogenates was determined using the bicinchoninic acid (BCA) assay (Smith et al., 1989).

Cell Culture: For each cell line, HT-29 and HCT 116, approximately $1x10^6$ cells were seeded into each of ten, 100 mm

diameter polystyrene tissue culture plates. Cells were seeded in McCoy's 5A medium supplemented with 10% FBS plus antibiotics. After 48 hours of culture, the medium was removed from plates, plates were rinsed once with PBS, and fresh McCoy's 5A medium containing 0.1% FBS and antibiotics was added. Cells were maintained in this low serum medium for 48 hours, at which time the majority of cells would be expected to be in G_0/G_1 phase of the cell cycle. The serum deficient medium was sufficient to maintain cell survival, but not sufficient to stimulate cell proliferation. After 48 hours in the serum deficient medium, cells from two plates for each cell line were harvested in lysis buffer. These cell lysates formed the 0 hour samples. The medium in the remaining eight plates for each cell line was switched to McCoy's 5A medium containing 10% FBS and antibiotics. Cells from two plates per cell line were harvested in lysis buffer at 0.5 and 8 hours after switching to medium containing 10% FBS. This experiment was replicated thrice.

Cell lysates were combined for each pair of duplicate plates at each time point. The lysates were allowed to stand on ice for thirty minutes and were then transferred to 1.5 ml microcentrifuge tubes and centrifuged for 10 minutes at 14,000 rpm to obtain a clear supernate that was used for further analysis.

Polyacrylamide Gel Electrophoresis (PAGE): The protein

homogenates collected from study 1 and 2 were combined 1:1 with SDS sample buffer and separated on 12% or 10% accrylamide gels. The gels were stained with a half hour immersion in 0.2% solution of brilliant coommassie blue followed by several changes of destaining solution. High and low molecular weight markers were also run on the same gels.

2-Dimensional Electrophoresis (2-DE): Changes in phosphorylation of cellular proteins were studied using 2-DE followed by western blotting. 2-DE was conducted as described by Dunn and Burghes (1983) and in accordance with the instructions provided by the electrophoresis equipment supplier (Hoefer Scientific instruments; San Fransisco, CA). The first dimension was an isoelectric focusing (IEF) tube gel run in 7.5 cm long glass tubes (ID-1.5mm) using a pH gradient of 3-10. The samples (30 ug of protein) were focussed for 4 hours at a constant voltage of 500 Volts. At this time the tube gels were removed from the glass tubes and equilibrated for fifteen minutes in equilibration buffer (10% glycerol, 4.9 mM DTT, 2% SDS, trace of bromophenol blue, 0.125 M tris base, pH 6.8). The second dimensional separation(SDS-PAGE) was carried out in 8x10 cm mini-gels of 1.5 mm thickness (10% total acrylamide, 2.7% bis). The tube gels were annealed to the slab gels with agarose (1% solution in tank buffer). Mouse IgG F_c fragments were loaded in a well adjacent to the tube

gel and served as a marker for the electrophoresis, electrophoretic transfer and western blotting. Electrophoretic separation was carried out at a constant current of 80 mA.

Western Blotting: After the 2nd dimension electrophoresis, gels were equilibrated in Towbin's transfer buffer [25 mM tris, 192 mM glycine, 20% methanol] for 15 minutes at 25 C. Electrophoretic transfer was carried out in wet conditions using a Hoefer transfer unit. Transfers were conducted overnight in Towbin's transfer buffer at a constant current of 100 mA. The transfer unit was cooled by circulating cold water.

Visualization of phospho-tyrosine containing proteins : After electrophoretic transfer, the PVDF membranes were allowed to air-dry to facilitate protein adherance to the membranes. The membranes were rewetted with 100% methanol followed by distilled water before incubating in blocking buffer [3% BSA in wash buffer [0.1% tween-20 in PBS]] overnight at 4°C. This was followed by a two hour incubation with anti-phosphotyrosine monoclonal antibody (dilution 1:1000) in blocking buffer. After three washes of ten minutes each in wash buffer, the membranes were incubated with goat anti-mouse IgG-horseradish peroxidase conjugate for one hour. Membranes then were washed once for fifteen minutes and four times for five minutes each in wash buffer. Phosphotyrosine

containing proteins were detcted using Enhanced Chemiluminescence (Renaissance Western Blot Reagent; DuPont NEN Research Products). Emitted signal was captured on Reflection autoradiograghy film (DuPont NEN).

Analysis of Gels and Autoradiographs: The densitometric analysis was a densitometric program called ImageQuant. The analysis was done by a combination of this program and careful visual examination.

RESULTS AND DISCUSSION

Study 1. The hyperproliferative changes produced in the rat colon after administration of a colon carcinogen like DMH or azoxymethane mimic the early stages of development of colon cancer (Deschner and Maskens, 1982). The purpose of this study was to identify proteins whose level of expression increased or decreased in response to DMH, to be used as markers for the risk for colon cancer. For this the rats were administered DMH. Control rats were not given DMH. The colon mucosal proteins were extracted from each of the two treatments and analyzed by SDS-PAGE and visualized by coomassie blue stain. Approximately forty bands were visualized in this manner. The protein bands that changed in intensity between the two treatments, namely, DMH and no DMH administration had molecular weights in the range of 30 to 45 KDa (Figure 1.). These changes in the protein levels, however, were inconsistent between animals. Therefore, we concluded that another study had to be conducted to look at changes in colon mucosal protein levels resulted from DMH administration.

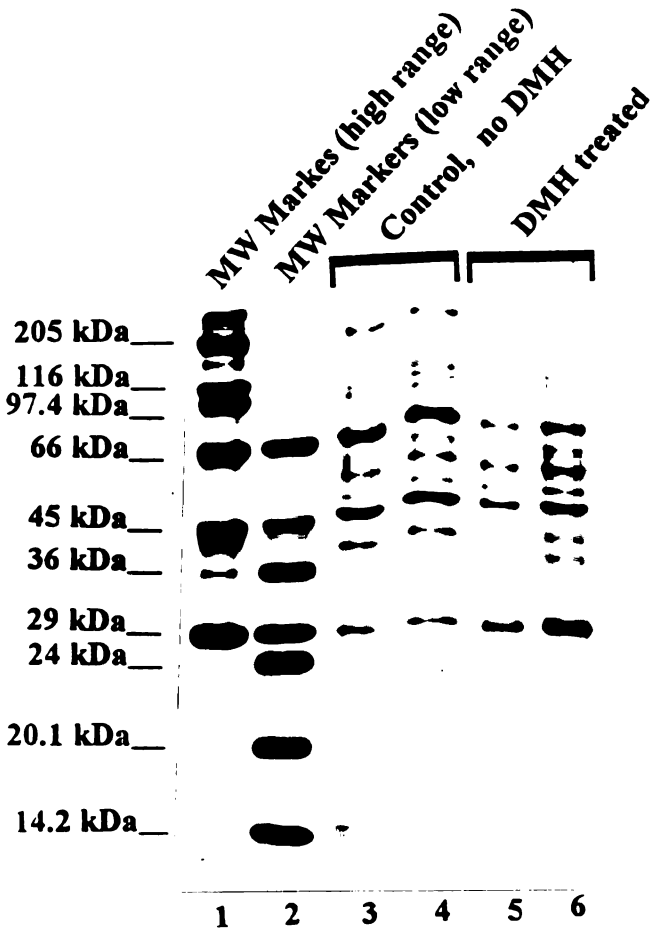

Figure 1. Proteins from colonic mucosal homogenates from control rat (no DMH treatment) and DMH treated rats resolved on 12% acrylamide SDS-PAGE gels. The proteins were stained with coomassie blue.

Study 2. From the review of the literature regarding induction of colon cancer by DMH administration it appears that the proliferation state of colon mucosa, expressed as labeling index (LI), changes over a period of several days after the carcinogen administration (Deschner, 1978). There is an initial increase in proliferative activity in response to the widespread cytotoxicity, a result of chemical administration. This is reflected by the increase in labelling index on days immediately following the carcinogen administration. The labelling index has been reported to decrease to a value closer to the baseline value after a few days (Deschner, 1978). This study was conducted to determine if the change in level proteins suggested in study 1 were transient or sustained over a few days. Five rats were used for this study, four of which were administered DMH. The control rat was not given DMH. In this study the rats receiving DMH were sacrificed on the third and fifth day after carcinogen administration. The mucosal proteins were extracted and analyzed as they were in study 1. Each lane of the SDS-PAGE contained equal amounts of protein. Figure 2 shows a gel from this study in which the proteins have been stained with coommassie blue. It was observed that the amount of proteins with molecular weights in 30 KDa to 45 KDa range changed relative to control on the third and the fifth day after DMH

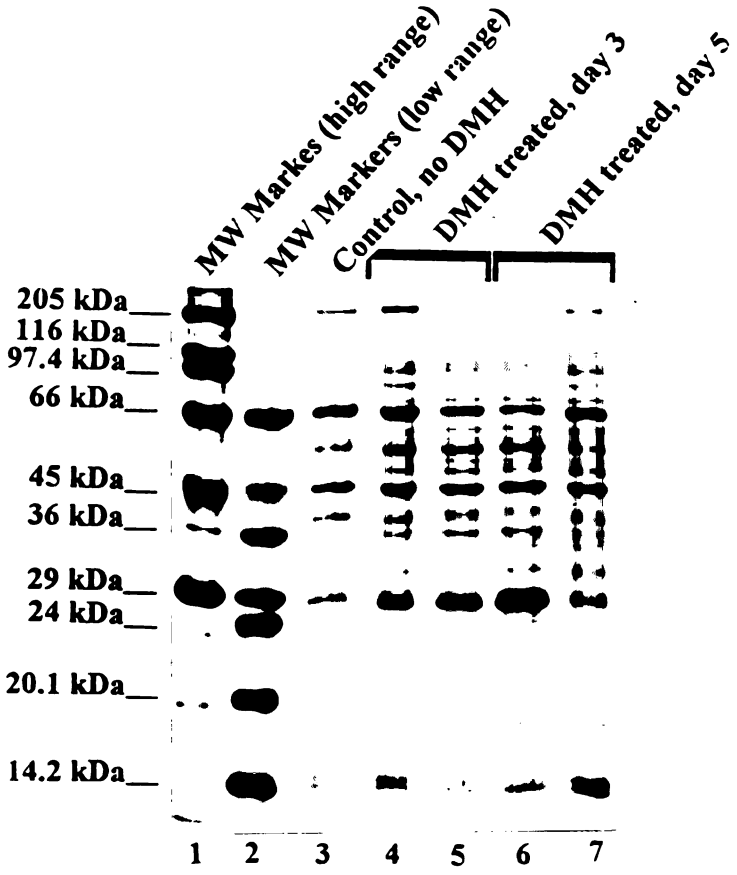

Figure 2. Proteins from colonic mucosal homogenates from control rat (no DMH treatment), DMH treated rats sacrificed on day 3 after treatment and DMH treated rats sacrificed on day 7 treatment resolved on 12% acrylamide SDS-PAGE gels. The proteins were stained with coomassie blue.

administration. However, any definite conclusions about the identity of protein bands of interest could not be drawn from this study. This was partly because of the large number of bands observed in the coomassie stained gel that caused overlapping of protein bands because of insufficient resolution.

In the course of these experiments, the significance of kinases and phosphatases in cell growth and division became more apparent in view of many of the oncogenic proteins being found to have kinase activity. It was realized that noticeable changes in response to mutagenic mutations might occur in the extent of phosphorylation of certain key proteins and not in the level of the proteins which we had earlier attempted to detect by coommassie staining. In view of this information, we decided to look at changes in tyrosine phosphorylation of colon mucosal proteins as a result of DMH administration. Therefore, the next two experiments focussed on studying tyrosine phosphorylation of proteins using western blotting.

The problem of poor resolution using SDS-PAGE was solved by switching to 2-dimensional electrophoresis (2-DE). 2-DE separates proteins according to their molecular weights and their isoelectric points which allows for a higher degree of resolution. In the first dimension the proteins are isoelectrically focussed in a tube gel. The second dimension

is run perpendicular to the first and achieves separation according to molecular weights. 2-DE has never been used to detect total changes in protein tyrosine phosphorylation in the proteins of the colonic mucosa.

Study 3. A cell culture study was conducted for this purpose. HT-29 and HCT 116 cells were grown in culture. The cells were serum deprived for 48 hours which arrests the cells in G_0 because the serum deprived media will support cell survival but does not have enough growth factors for the cells to continue dividing. The 0 hour collection was made at this point. The media was then replaced by serum replenished media to provide necessary growth factors and nutrients. At this point the cells proceed through the next mitotic cycle by intensively synthesizing DNA and proteins. The signal for the onset of DNA and protein synthesis travels from the plasma membrane to the nucleus, the site of nucleic acid synthesis. The binding of the growth factors to their respective receptors activate the kinases associated with the receptors or the membrane. This initiates a cascade of kinase and phosphatase activities. These events occur rapidly, therefore, the next sample was taken 0.5 hr after the media was switched. It was assumed that the phosphorylation and dephosphorylation

that occurred in the first half hour would be seen in this sample. The next sample was taken eight hours after serum replacement. The cells were all collected in lysis buffer. Equal amounts of protein as determined by BCA protein estimation assay were analyzed with 2-DE. The level of expression of proteins was determined by silver staining the gels. The tyrosine phosphorylation of proteins was determined by western blotting followed by immuno-probing with anti-phospho-tyrosine monoclonal antibody. The silver stained gels and phospho-tyrosine autoradiographs were analyzed using a densitometer. The density of the spot in the silver stained gels and phospho-tyrosine autoradiographs was taken to be indicative of the level of expression of the protein and the level of tyrosine phosphorylation of the protein respectively.

Approximately thirty to thirty-five phospho-tyrosine containing proteins were detected by this method in HT-29 and HCT 116 cells. The total number of proteins detected were about seventy. These were visualized by silver staining. The phosphotyrosine containing proteins that were observed to increase or decrease in the autoradiographs were carefully examined in the silver stained gels. This was to assure that the change observed was due to change in the tyrosine phosphorylation state of the protein and not due to change in the level of expression of the protein. Since the experiment was conducted in triplicate, there are three sets of values

for each cell line. The variation in the intensity of the protein spots between triplicates was quite large. Only the spots that changed in a consistent manner between the replicates are reported in Tables 2 and 3. The results are reported as intensity of protein spots as compared to the intensity of the particular spot in control (time, 0 hour)(Table 2 and 3). These results have been obtained by a careful visual examination of the protein spots. An accurate densitometeric evaluation could not be obtained due to the varying levels of background found in the different parts of the gels which cannot be excluded from the spot when demarcating the area.

The number and clarity of the protein spots observed with 2-DE was much greater than that obtained with PAGE and clearly justified the use of 2-DE, which is more time and labor intensive than PAGE. The approximate molecular weights and approximate isoelectric points were calculated from the relative distance traveled by a certain protein in each perpendicular direction and are also reported in the Tables 2 and 4. The only spots reported here are the ones that did not change in intensity in the silver stained gels. Therefore, the changes observed are due to changes in protein tyrosine phosphorylation and not due to the difference in the level of the protein present in cell.

HT-29 The 50 kDa protein of approximate isoelectric point 6.4 was not detected in the first replicate. In the second replicate of the experiment, it was detected only in the half hour cells. In the third replicate, however, it was detected at all three time points. The intensity of the spot in the phosphotyrosine autoradiograph for one half hour cells was greater than the 0 hour and the eight hour cells. This pattern suggests that the 50 kDa protein is transiently phosphorylated at the onset of the mitotic cell cycle after which it reverts to it's baseline level of tyrosine phosphorylation.

The 56 kDa protein of approximate isoelectric point 5.7 was observed to decrease at the half hour time point in all three replicates compared to the controls at 0 hour. At eight hours this phospho-tyrosine spot increased to either the baseline control level or even higher. This protein appears to be dephosphorylated for a short time at the beginning of the cell cycle.

Another 56 kDa protein with an isoelectric point of 5.5 was observed to be tyrosine phosphorylated to a greater extent at the half hour time point compared to the 0 hour control. In the first and the second replicate the phosphorylation seemed to return to the baseline control level, but stayed elevated in the third replicate. This could indicate a protein which stays at the higher level of tyrosine phosphoryaltion for

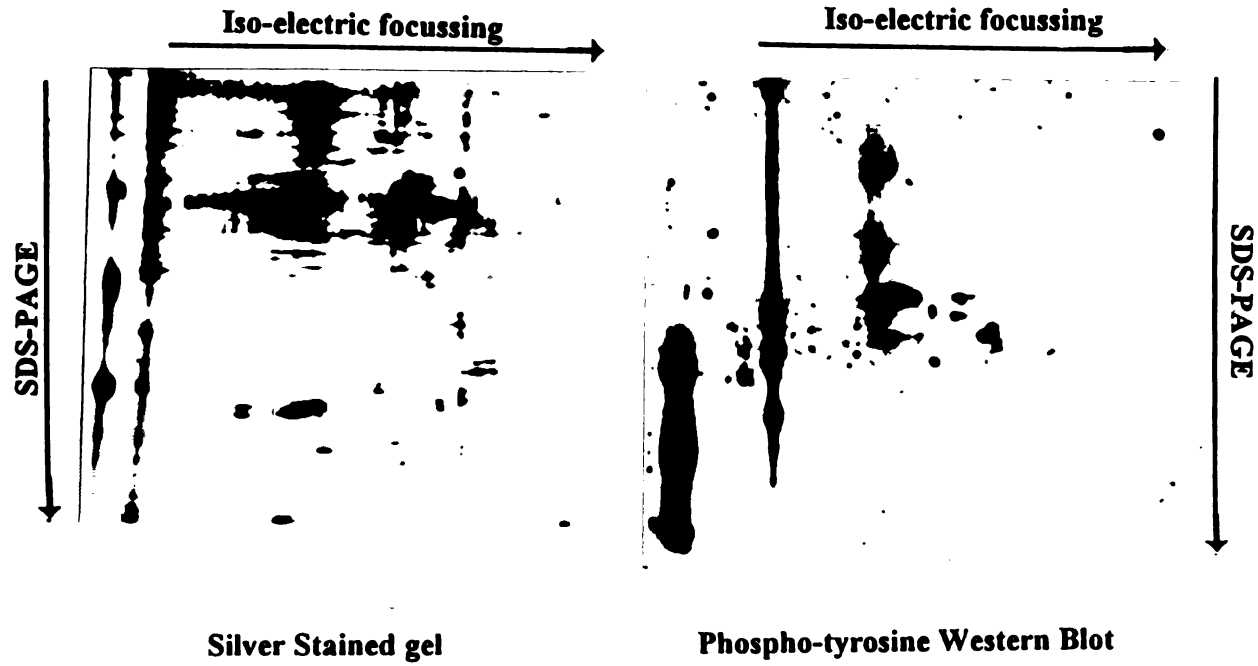

Figure 3. Proteins from HT-29 cells 0 hrs after stimulation by the addition of FBS to the media, resolved by 2-dimensional electrophoresis and stained with silver stain (left), and autoradiograph from a western blot of phosphotyrosine (right).

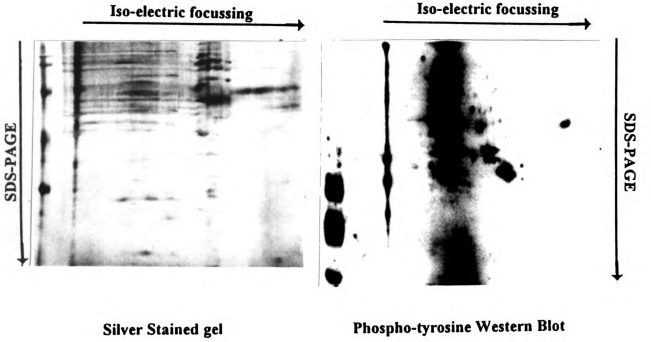

Figure 4. Proteins from HT-29 cells 0.5 hrs after stimulation by the addition of FBS to the media, resolved by 2-dimensional electrophoresis and stained with silver stain (left), and autoradiograph from a western blot of phosphotyrosine (right).

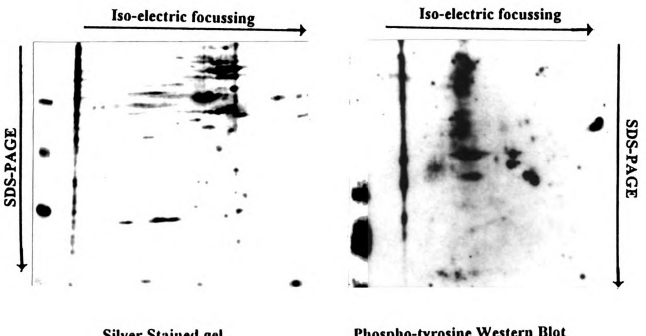

Figure 5. Proteins from HT-29 cells 8 hrs after stimulation by the addition of FBS to the media, resolved by 2-dimensional electrophoresis and stained with silver stain (left), and autoradiograph from a western blot of phosphotyrosine (right).

Table 2. Phosphotyrosine containing proteins in HT-29 cells. The three symbols within each replicate represent three time points, 0 min, 30 min and 8 hrs after addition of FBS.

MWkDa (SD)	pI (SD)	1st Replicate			2nd Replicate			3rd Replicate		
		0 min	30 min	8 hr	0 min	30 min	8 hrs	0 min	30 min	8 hrs
48.9(2.3)	6.4(0.0)				-	P	-	P	>	P
56.8(0.7)	5.7(0.2)	P	<	P	P	<	>	P	<	P
56.8(0.7)	5.5(0.1)									
77.6(2.0)	3.8(0.1)	P	>	P	P	>	P	P	>	>
					-	P	P	-	P	P

Key : - Not present at detectable level
P Present at detectable level
> Present at level greater than control
< Present at level lesser than control

longer period than the two proteins described earlier.

The 75 kDa protein with isoelectric point 3.8 was only detected in the second and third replicates. In the second and third replicates, this spot was not detected at 0 hour time point. At the half hour and eight hours time points the level of tyrosine phosphorylation appears to remain constant. This protein apparently is not phosphorylated at it's tyrosine residues when the cell is in the non-dividing state. But once the cell starts cycling, it stays phosphorylated for a long period of time.

HCT 116 The proteins discussed here for HCT 116 cells are tabulated in Table 3. The 40 kDa protein with isoelectric point 6.1 appears to increase in intensity at half hour time point compared to 0 hour control in phospho-tyrosine autoradiographs. It was observed to stay elevated in the first and the third replicates, but seemed to return to baseline eight hour level in the second replicate.

The 42 kDa protein with isoelectric point 6.6 was not detected in the first replicate. In the second and third replicate, the intensity of the spot in the phospho-tyrosine autoradiographs was observed to be increased at the half hour time point as compared to the 0 hour control. At the eight hour time point, the level of phosphorylation seemed to return

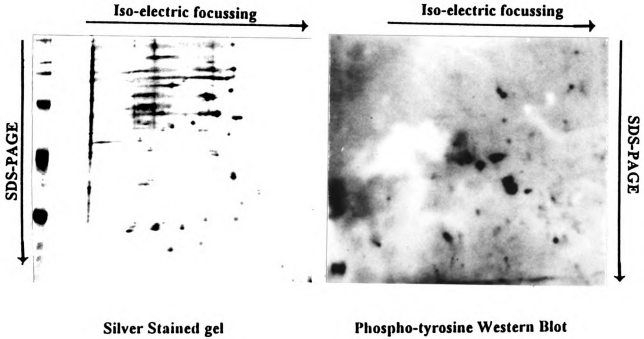

Figure 6. Proteins from HCT 116 cells 0 hrs after stimulation by the addition of FBS to the media, resolved by 2-dimensional electrophoresis and stained with silver stain (left), and autoradiograph from a western blot of phosphotyrosine (right).

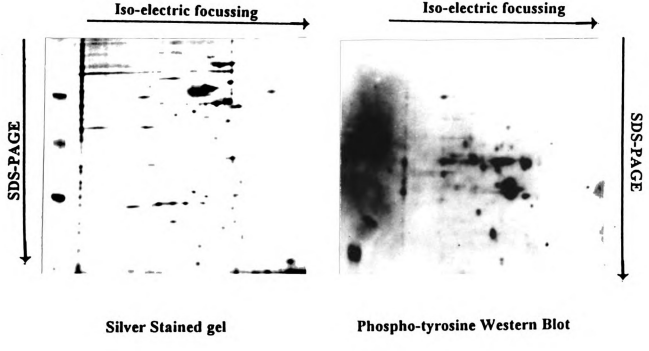

Figure 7. Proteins from HCT 116 cells 0.5 hrs after stimulation by the addition of FBS to the media, resolved by 2-dimensional electrophoresis and stained with silver stain (left), and autoradiograph from a western blot of phosphotyrosine (right).

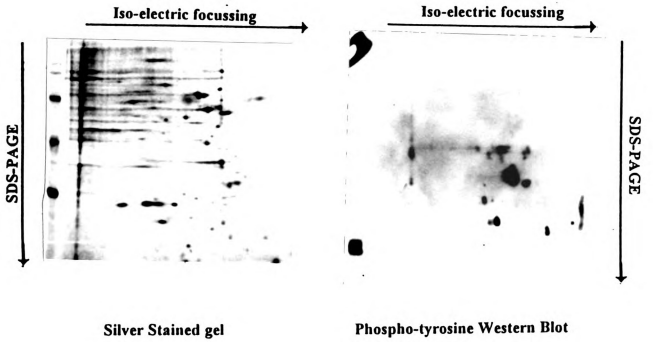

Figure 8. Proteins from HCT 116 cells 8 hrs after stimulation by the addition of FBS to the media, resolved by 2-dimensional electrophoresis and stained with silver stain (left), and autoradiograph from a western blot of phosphotyrosine (right).

Table 3. Phosphotyrosine containing proteins in HCT 116 cells. The three symbols within each replicate represent three time points, 0 min, 30 min and 8 hrs after addition of FBS.

MW kDa (SD)	pI (SD)	1st Replicate			2nd Replicate			3rd Replicate		
		0 min	30 min	8 hrs	0 min	30 min	8 hrs	0 min	30 min	8 hrs
28.7 (9.9)	5.4 (0.2)	P	P	>	-	P	-	-	P	P
41.1 (4.1)	6.1 (0.1)	P	>	>	P	>	P	-	P	P
42.1 (3.8)	6.6 (0.0)				P	>	P	-	P	P
42.9 (5.6)	4.9 (0.1)	-	P	-	-	P	-			
51.0 (4.4)	6.5 (0.1)	P	>	P	P	>	P	-	P	P
58.7 (7.0)	5.5 (0.1)	-	P	-	P	>	-	-	P	P
59.9 (11.1)	5.0 (0.0)	-	P	P	-	P	P			
60.2 (4.0)	4.7 (0.3)	-	P	-	P	>	P	-	P	P
70.0 (4.7)	7.5 (0.0)				-	P	<	-	P	P
92.1 (3.8)	4.4 (0.1)	-	P	-	P	>	P	-	P	P

Key : - Not present at detectable level
P Present at detectable level
> Present at level greater than control
< Present at level lesser than control

to baseline in replicate II, but seemed to stay elevated in replicate III.

The 43 kDa protein with isoelectric point 4.9 was not detected in replicate III. In the first and the second replicate, this protein was only detected at the half hour time point. It appears to be a protein that is only transiently tyrosine phosphorylated at the onset of the cell division.

The 51 kDa protein with isoelectric point of 6.5 was observed to have increased tyrosine phosphorylation at half hour time point compared to 0 hour time point in all three replicates. At eight hours the level of tyrosine phosphorylation seemed to decrease to baseline levels in the first and the second replicates, but seemed to stay elevated in replicate III.

The 58 kDa protein with approximate isoelectric point 5.5 was phosphorylated at tyrosine residues to a greater extent compared to 0 hour control. At eight hours, though, the level of phosphorylation differed in all three replicates. In the first replicate, the intensity of the spot seemed to return to the baseline level, which was undetectable in this replicate. In the second replicate, the level of tyrosine phosphorylation decreased lower than detectable levels at eight hours. In the third replicate the level of tyrosine phosphorylation remained

constant at the half hour and the eight hour period.

The 59 kDa protein with approximate isoelectric point 5.0 was not detected in the third replicate. In the first and second replicate, however, the tyrosine phosphorylation spot increased in intensity at the half hour time point and stayed elevated at the eight hours time point.

The 60 kDa protein has an approximate iso-electric point of 4.7. In all replicates the level of tyrosine phosphorylation increased at the half hour time point compared to 0 hour control. In the first and the second replicate the level of tyrosine phosphorylation seemed to decrease to the level of the 0 hour control at the 8 hr time point, except in replicate III where the tyrosine phosphorylation appeared to stay constant over the eight hour period.

The 70 kDa protein with iso-electric point of 7.5 was not detected in the first replicate. In the second and the third replicate the level of tyrosine phosphorylation increased from undetectable levels to detectable levels at the half hour time point. At eight hours it stayed detectable in both replicates II and III, however, the level though stayed constant in the third replicate and decreased slightly in the second replicate.

The 92 kDa protein with iso-electric point 4.4 was tyrosine phosphorylated at increased levels at half hour time

periods compared to 0 hour control in the three replicates. At eight hours the levels of tyrosine phosphorylation seemed to come down to the baseline levels in replicates I and II. However, the level of tyrosine phosphorylation appeared to remain constant at the eight hour period in replicate III.

Study 4. The next study was conducted with ten rats. Of these, four were controls with no DMH treatment, three were sacrificed on day three after a single DMH injection and other three were sacrificed a week after the DMH injections (Table 4). Approximately ninety protein spots were detected in the phospho-tyrosine autoradiographs. The approximate molecular weights and isoelectric points of these proteins were calculated using the R_f values and comparing them to those for the markers. There was a large variation in the patterns of the appearance and disappearance of phospho-tyrosine spots.

The 36 KD protein with approximate iso-electric point 4.35 was present at a higher level in the three rats at day three after the DMH injection. But on day seven, it had decreased to control levels.

The 37 kDa protein with iso-electric point 4.4 followed the same pattern as described for the 36 kDa protein. The protein showed a much darker spot in all third day rats compared to the control and the seventh day rats.

Table 4. Phosphotyrosine containing protein spots in DMH treated and control rats. The three/four symbols in each group represent three/four rats in that treatment group.

MW (kDa)	pI	Control	DMH-day	DMH-day 7
36	4.3	P P -	> > >	P P P P
37	4.4	P P -	> > >	P P P P
42	5.3	- P -	> > >	- - - -
44	5.4	- P -	> > >	- - - -
54	5.0	- P -	> > >	> > > P
67	6.5	P P P	P P P	< < < <
67	6.2	P P P	- - -	- - < <

Key : - Not present at detectable level
P Present at detectable level
> Present at level greater than control
< Present at level lesser than control

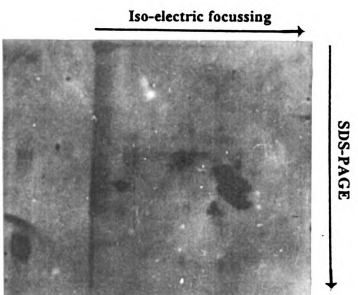

Figure 9. Colonic mucosal proteins from a control(no DMH administration) rat, resolved by 2-dimensionanl electrophoresis, and western blotted for phosphotyrosine.

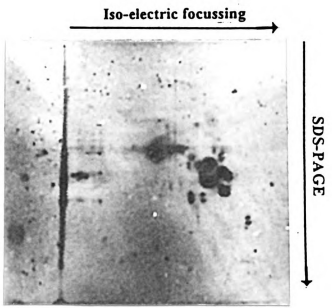

Figure 10. Colonic mucosal proteins from a DMH treated rat (sacrificed day 3 after administration), resolved by 2-dimensional electrophoresis, and western blotted for phosphotyrosine.

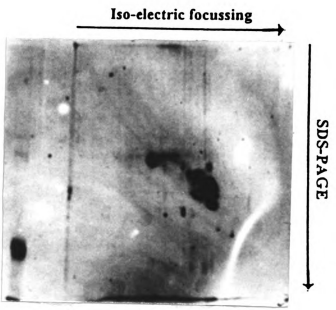

Figure 11. Colonic mucosal proteins from a DMH treated rat (sacrificed day 7 after administration), resolved by 2-dimensional electrophoresis, and western blotted for phosphotyrosine.

Table 5. Phospho-tyrosine containing proteins common to HT-29 and HCT 116.

MW (kDa)	pI	Level of Phospho-Tyrosine		
		0 min	30 min	8 hrs
50	6.4	P	>	P
57	5.5	P	>	P

Key : P Present at detectable levels
> Present at levels higher than control

The 43 kDa protein had an iso-electric point of 5.4 was present in undetectable levels in two of the three control rats. On the third day after DMH treatment the level of the tyrosine phosphorylation of the protein was elevated in all three rats. The protein was not detected in phospho-tyrosine autoradiographs in seventh day rats, presumably because the tyrosine phosphorylation was below detectable levels.

The 44 kDa protein with iso-electric point 5.4 was detected only in the third day rats, with the exception of one rat in the control group. Seemingly this protein was tyrosine phosphorylated in response to carcinogen administration but did not remain so for a very long time.

The 54 kDa protein of iso-electric point 5.0 was the only protein in this study that exhibited elevated tyrosine phosphorylation on day three and stayed elevated on day seven of the experiment.

The 67 kDa protein with iso-electric point 6.5 was present at a constant level of tyrosine phosphorylation in control and third day rats. The tyrosine phosphorylation seemed to decrease on day seven of the experiment in all four rats in this group.

The 67 kDa protein with iso-electric point 6.2 was not detected in phospho-tyrosine autoradiographs for third day rats. In the seven day rats it was detected only in two of the four rats where it was present at a lower density than the

control rats in the phospho-tyrosine autoradiographs. This protein, therefore, appears to be dephosphorylated and stay dephosphorylated, as a result of carcinogen administration.

The large variation in tyrosine phosphorylation of proteins can be explained on the basis that the cells of the colon mucosa in a live animal, unlike the cells grown in culture, are a mixture of cycling cells and are not synchronized in terms of cell cycle. In addition to that, there is also a diurnal effect in terms of proliferation, i.e., some cells are always dividing in the tissue, but the fraction of cells diving at certain times of the day are higher than the others. Therefore, at any given time, the mucosal cells are in various phases of the cell cycle. The extent of tyrosine phosphorylation of a protein probably depends on the fraction of total cells present in the phase of cell cycle in which that protein is tyrosine phosphorylated.

The pattern of increase or decrease in phosphorylation of mucosal proteins seen over a period of several days can be important in assessing the validity of that protein as a marker for hyperproliferation. The proteins that are phosphorylated for a short period of time, almost transiently, suggests a regulatory role for these proteins in the cell cycle. These proteins are probably placed at crucial branch points of the cell regulatory pathways and are only required to be phosphorylated or dephosphorylated transiently. During

the short interval of it's being active, this protein probably activates other downstream proteins. The proteins that are phosphorylated or dephosphorylated for longer periods of time probably have a functional role, for which they are required to be in the modified or active form for the duration of the time the cell takes to divide.

The proteins that are phosphorylated or dephosphorylated for longer periods of time show a greater potential for being used as a marker for dividing cells as there is a greater possibility of these being detected. On the other hand, though, the changes in phosphorylation of these proteins may just be downstream event of mutations in the regulatory proteins and thus might be seen later than regulatory proteins. Changes in phosphorylation of the proteins regulatory may be, therefore, a more sensitive marker for proliferation.

From a careful examination of the molecular weights of the proteins identified in this study, some suggestions can be made regarding the identity of these proteins. These suggestions are based on the comparisons between the molecular weights of the proteins. Only the proteins that have been implicated in the regulation of the proliferation of cells or cell division have been considered. Most of these proteins are either known to be tyrosine phosphorylated under physiological conditions or a strong possibility of such post-translational

modification exists.

Bcl-2, a 26 kDa protein, is phosphorylated, an event coincident with it's association with $p21^{Ras}$. Inhibition of this phosphorylation prevents protection from apoptosis by Bcl-2. In other words, phosphorylated Bcl-2 provides protection from apoptosis in Jurkat cells (Chen and Faller, 1995). The molecular weight of this protein corresponds to p28 identified in HCT 116 cells.

$p34^{cdc2}$ forms a complex with cyclin B, that though indetectable at interphase, rises abruptly at G_2/M transition to induce mitosis. After the synthesis of cyclin B, the suppression of the complex has been attributed to the phosphorylation of $p34^{cdc2}$. When cyclin B accumulates to a critical threshhold level, it sets off a cascade of events that leads, after a lag period, to a switch in the balance between kinases and phosphatases such that the inhibitory phosphorylations on $p34^{cdc2}$ are suddenly removed. The 36 kDa and the 37 kDa protein identified as having increased tyrosine phosphorylation in response to DMH administration in rats fall within the molecular weight range of $p34^{cdc2}$. The probability of it being the same protein seems limited, however, in view of the fact that the tyrosine phosphorylation of $p34^{cdc2}$ would be expected to go down in response to DMH, a stimulator of proliferation, whereas the p36 and p37 seem to have a higher level of tyrosine phosphorylation.

The human Cdk Activating protein Kinase 1(CAK 1) is reported to be a cdc-2 related cyclin-dependent protein kinase with a molecular weight of 42 kDa. If this protein is regulated in a manner similar to other cdc2 related proteins, it would be expected that it associates with other regulating partners, that is, cyclins and is is further regulated by site specific phosphorylation or dephosphorylation. Microsequence analysis after limited proteolysis has confirmed that CAK does bind p35, a protein that demonstrates a great deal of similarity to cyclin H. The two 42 kDa proteins identified in HCT 116 cells and the 42 kDa protein identified in rats could be the same protein as p42 CAK.

In addition, the p42 MAP kinase is known to be tyrosine phosphorylated and activated in the T cell lymphoma cell line 171CD4+ when the antibody binds to the CD4 surface antigen (Ettehadieh et al., 1992). The p42/p44 MAPK kinaes have been identified by several groups (Gomez and Cohen, 1991; Rossomando et al., 1992; Ahn et al., 1991). The MAPKK is a 45-46 kDa dual specificity protein, that autophosphorylates tyrosine and threonine residues on itself in response to activation by v-Raf (Dent et al., 1992).

p57 is a cytoplasmic protein found in colon carcinoma cells that has recently been identified as a new MAP kinase (Lee et al., 1993b) or an extracellular signal regulated kinase (Rossomando et al., 1989; Boulton et al., 1991;

Kyriaskis and Avruch, 1990). Stimulation of undifferentiated colon carcinoma cells with DAGs diolein or dimyristin or with TPA leads to phosphorylation of tyrosine on p57 within 1-2 minutes (Lee et al., 1993b; Marian et al., 1989). Signal transduction through p57 has also been reported to involve c-src in colon carcinoma cells (Lee et al., 1993b) which can be expressed at higher levels in colon cancer (Aaronson, 1991). The two 56 kDa proteins identified in HT-29 cells and the 58 kDa protein in HCT 116 cells could possibly be the same as p57.

The 60 kDa protein identified in HCT 116 cells as having decreased levels of tyrosine phosphorylation after rising briefly lies within the molecular weight range of $pp60^{c\text{-}src}$. Dephosphorylation of Tyr-527 is required for the activation of Src (Cooper et al., 1986) and src has been reported to be activated in human colon carcinoma (Bolen et al., 1987).

In mouse NIH-3T3 cells transformed with an activated allele of c-src in which the regulatory tyrosine is replaced by phenylalanine, tyrosine phosphorylation of a 68 kDa protein was found to be greatly enhanced (Fumagalli et al., 1994). Purification and microsequencing of p68 showed that it was related to the p62 GAP associated protein that has been described before (Ellis et al., 1990). It appears that during mitosis src binds via it's SH2 and SH3 domains to tyrosine phosphorylated p68 (Park and Cartwright, 1995). The two 67 kDa

proteins identified in rat colon to be tyrosine phosphorylated have the possibility to be the same as p68.

SUMMARY AND CONCLUSIONS

Hyperproliferation, an early bio-marker for colon cancer, is presently detected in histological samples by immunohistochemistry or radio-labeling. This study was conducted to look for tyrosine phosphorylated proteins that could be used as markers for hyperproliferation. Dimethylhydrazine administration was used to induce hyperproliferation in the colon mucosa of rats.

Synchronized HT-29 and HCT 116 cell cultures were used to detect changes in tyrosine phosphorylation in cells at different stages of the cell cycle. In HT-29 cells, two proteins of molecular weights 50 and 56 KD were found to be tyrosine phosphorylated to a greater extent at the 0.5 hr time point compared to the 0 hr and 8 hr time points. Another 56 KD protein was less tyrosine phosphorylated at 0.5 hr time point compared to the 0 and 8 hr time points and a 77 KD protein was tyrosine phosphorylated to a detectable level only at 0.5 and 8 hr time points.

In HCT 116 cells, five proteins with approximate molecular weights 43, 51, 58, 60 and 92 KD were found to be

more tyrosine phosphorylated at 0.5 hr time point compared to the 0 and 8 hr time points. The 41 and the 59 KD proteins were phosphorylated to a greater level at 0.5 as well as 8 hr time points compared to 0 hr time point.

Two proteins were identified that behaved in identical fashion between the two cell lines. These proteins were of molecular weights of approximately 50 and 57 KD.

It was concluded from this study that 2-DE followed by western blotting followed by probing the membranes with anti-phospho-tyrosine was an effective technique to detect changed tyrosine phosphorylation of total proteins of cultures colon cancers cells, HT-29 and HCT 116's.

For the next study, rats were given a single injection of DMH and were sacrificed on the third and seventh day after the injection. The control (no DMH) rats were sacrificed on third day after the injection. The DMH injection resulted in increased tyrosine phosphorylation compared to control on day three, but not day seven in four proteins of approximate molecular weights 36, 37, 42 and 44 KD. A 54 KD protein was more tyrosine phosphorylated compared to the control on day three and seven after injection. A 67 KD protein was found to be less tyrosine phosphorylated on day seven compared to day three and control. Another 67 KD protein was found to tyrosine phosphorylated at much lower level on day three and seven

after the injection compared to control.

RECOMMENDATIONS FOR FUTURE RESEARCH

1. Present work has suggested several proteins whose tyrosine phosphorylation could potentially be used as markers for hyperproliferation in colon mucosal tissue. Possibly some of these proteins are related to the cell cycle regulatory proteins. In recent years many of these proteins have been identified and monoclonal antibodies against them are commercially available. In future research these antibodies could be used to identify the proteins indicated by present study.

2. Since this work has exhibited that 2-DE can be used to detect changes in protein tyrosine phosphorylation, this ability should be used to test for the effect of certain alleged tyrosine kinase inhibitors, such as genistein in diet, on tyrosine phosphorylation in tissues. Genistein, an isoflavanoid found in some foods, e.g., soybeans, has been reported to inhibit cell proliferation, but the mechanism by which it does so is not completely understood. One of the possible mechanisms suggested for it's action is by inhibition of tyrosine phosphorylation. This can be studied

using the technique used in this study.

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