THE EFFECT OF RETINOIDS ON THE GROWTH AND DIFFERENTIATION OF COLON CANCER CELLS

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The Effect of Retinoids on the Growth and Differentiation of Colon Cancer Cells

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ABSTRACT

Retinoids has been shown to induce differentiation in various cell types. Nevertheless, the mechanism of induction of differentiation by retinoids is not understood. This investigation was designed to study the effect(s) of retinoic acid (RA) on the growth of human colon carcinoma (HT-29) cells.

Our results demonstrated that retinoic acid inhibited the proliferation of HT-29 cells in vitro. Retinoic acid caused 17 to 82% inhibition of growth in a time and dose dependent manner. Maximum inhibition was achieved at 10^{-5} M 13-*cis*-RA 96 hours post treatment. In parallel experiments RA also decreased the level of ornithine decarboxylase activity by eight fold and ornithine decarboxylase mRNA levels by seven fold by the fifth day of treatment. On the other hand RA treatment resulted in a three fold increase in major histocompatibility (MHC) class I mRNA and four fold increase in protein kinase C activity by the fifth day, where as the levels of expression of both carcinoembryonic antigen and Ras remained the same. Further more the transcription factors AP-1 and Sp1 were down- and up-regulated respectively, during the duration of the treatment.

This study has shown that the effects of RA in HT-29 cells are mediated via the protein kinase C signal transduction pathway and the observed growth inhibition may be due to the decrease in ornithine decarboxylase. The increase in MHC class I mRNA and the non overlapping reciprocal regulation of expression of AP-1 and SP-1 transcription factors are suggestive of terminal differentiation and a return to phenotypic normalcy. The findings presented in this work would contribute to the development of effective strategies for the treatment of colon cancer.

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CHAPTER 1

INTRODUCTION

Colon cancer has one of the highest incidences among cancers. Besides its high incidence, the high mortality rates place this disease as the second leading cause of deaths from cancer (Schwartz, 1990). Colon cancer develops from a sequential step of progressive alterations of normal colonic mucosal cells through a polyp (adenoma phase) before becoming malignant (Cho and Vogelstein, 1992). These genetic alterations include somatic activation of cellular oncogenes through point mutation, amplification and the germline or somatic inactivation of tumor suppressor genes through point mutation or deletion or both (Vogelstein et al., 1988). This model is supported by studies of human colorectal tumors demonstrating that the progressive accumulation of genetic changes in both oncogenes and tumor suppressor genes parallels the clinical progression (Monpezat et al., 1988; Nishisho et al., 1991; Yokota et al., 1986; Erisman et al., 1985; Bos et al., 1987).

Given the significance of colon cancer as a major health problem, recent efforts seek to develop therapeutic alternatives to the conventional ones. A new approach to cancer prevention and non-cytotoxic therapy is the use of differentiating agents (Michaeli et al., 1992). The basis for this approach is the demonstration that malignant phenotype can be suppressed or reversed by induction of differentiation or by restoration of responses to normal growth control mechanisms in cancer cells.

Retinoids, a family of molecules comprising both the natural and synthetic analogues of vitamin A, can modulate the growth and differentiation of normal, premalignant, and malignant epithelial and mesenchymal cells *in vitro* and *in vivo* (Lotan, 1980; Roberts and Sporn, 1984; Sherman, 1986; Amos and Lotan, 1990). The physiologic effects of retinoids *in vivo* include: regulation of developmental and morphogenetic

processes, prevention of squamous cell differentiation in nonkeratinizing epithelial tissues, control of differentiation of certain mesenchymal tissues, and regulation of certain aspects of the immune response (Lotan, 1980; Sherman, 1986; Dennert, 1984). Retinoids exert distinct effects on the growth and differentiation of various types of tumor cells *in vitro* (Lotan, 1980; Amos and Lotan, 1990; Lotan et al., 1981; Roberts and Sporn, 1984) and *in vivo* (Halter et al., 1988; Lippman et al., 1989). They enhance differentiation of embryonal carcinoma cells along endodermal, neuronal, or mesenchymal pathways, induce neuronal differentiation in neuroblastoma cells, myeloid differentiation in promyelocytic leukemia cells, monocytoid differentiation in premonocytic leukemia cells, erythroid differentiation in erythroleukemia cells, and melanocytic differentiation in melanoma cells (Roberts and Sporn, 1984; Amos and Lotan, 1990).

Other important features of retinoids are their ability to suppress carcinogenesis and inhibit tumor growth in experimental animals (Lotan, 1980; Roberts and Sporn, 1984; Sherman, 1986; Amos and Lotan, 1990), and their efficacy in chemoprevention and therapy of certain types of malignancy in humans (Lippman et al., 1989; Castaigne et al., 1990). Because of these properties, retinoids are considered one of the more promising candidates for 'differentiation therapy'.

One of the major effects of retinoids is their ability to regulate cellular proliferation of the cell cycle in which ornithine decarboxylase (ODC) is a key enzyme (Denhart et al., 1986). Several studies have reported elevated ODC activity in human colon cancers as well as in other cancers (Luk and Baylin, 1984; Guillem et al., 1990; Elitsur et al., 1992). However, the effect of growth inhibitors such as retinoids on ODC has not been shown.

Colon cancer cells and other malignant tumors escape the immune surveillance of the host by down regulating the expression of HLA class I recognition molecules on their surfaces (Tanaka et al., 1985). As a result, the host resistance fails to eliminate the abnormal cells. Major histocompatibility complex (MHC) class I antigens are involved in intercellular communication, and recognition by cytotoxic T cells (Klein, 1986). Thus

tumor cells that fail to express them have growth advantage. Studies have shown that MHC class I antigen levels are markedly reduced in various malignant tumors including colon tumors (Bar-Eli et al., 1988). The rationale for the study of MHC class I gene expression in retinoic acid-treated colon cells is to test whether retinoids can induce HLA class I expression in HT-29 colon carcinoma cells, there by making them susceptible to destruction by immune cells.

Carcinoembryonic antigen (CEA), a widely used human tumor marker, is expressed in most human carcinomas and in a high proportion in colon carcinoma cells and cells of the gastrointestinal tract (Shiveley and Beatty, 1985). The expression of the CEA is related to the degree of tumor differentiation and has been shown to be modulated by retinoids in several model systems (Niles et al., 1988). Therefore the determination of the level of CEA by retinoids is of significant value since it is used for the follow-up evaluation of colon carcinoma after drug or radiation therapy.

The regulation of cell division by growth factors, as well as cell transformation induced by several viral oncogenes, involves the activation of protein kinases (Nishizuka, 1986; Sposi, 1989). Calcium and phospholipid-dependent protein kinase C (PKC) is known to be associated with increased proliferation in several cell types including colonic epithelial cells (Sakanoue et al., 1991; Craven and DeRubertis, 1987). The evidence of *in vitro* studies suggested the possibility that activation and/or translocation of PKC from cytosolic to particulate fraction may serve as a positive intracellular signal to colonic epithelial cell growth in retinoid-mediated differentiation.

Sequence-specific DNA binding proteins are believed to play a key role in such vital cellular processes as transcription, replication and recombination. In transcription, these proteins regulate the rate of mRNA synthesis by binding to discrete DNA sequences termed promoters. The activity of the cell proliferation and differentiation related transcription factors may be regulated in a cell-specific or cell-cycle dependent manner as

well as responding to extracellular signaling. Regulation of AP-1 and Sp1 transcription factors are studied in retinoic acid induced inhibition of proliferation of colon cancer cells.

A human adenocarcinoma cell line HT-29 (Fogh and Trempe, 1975) was selected as an *in vitro* model to study the effects of retinoids on colon cancer. The cells are epithelial like, moderately differentiated, and have retained certain characteristics of normal colon tissue, such as hormone receptors. Their tumorigenic potential is well-documented (Trainer et al., 1988). Furthermore, HT-29 cells secrete various peptides, including factors stimulating the proliferation of fibroblasts (Anzano et al., 1989).

13-*cis* retinoic acid was selected to be used in all of the studies over the two other retinoids (retinol and all-*trans* retinoic acid) for its effectiveness in the inhibition of proliferation of the HT-29 colon cancer cells *in vitro*.

This study elucidates the effects of retinoic acid induced differentiation and proliferation in an *in vitro* model of human colon cancer investigating the expression of colon carcinoma associated oncogenes, cell surface markers, expression of the cell cycle specific enzyme ODC, activity of the signal transduction related enzyme PKC, and regulation of transcription factors AP-1 and Sp1. Results of this investigations should enhance our understanding of the molecular mechanisms of cellular differentiation and ultimately should contribute to the development of effective strategies for colon cancer treatment.

The specific aims of this study are; to investigate whether retinoic acid-induced inhibition of HT-29 growth is:

(1) related to altered expression of oncogenes; especially those that are implicated in colon carcinogenesis;

(2) related to the differential expression of cell cycle specific genes such as ODC whose product is a step limiting enzyme in polyamine synthesis leading to DNA replication which ultimately results in cell growth.

(3) related to the expression of MHC class I genes;

(4) related to changes in the expression of CEA that correlate with the state of differentiation in colon tumor cells;

(5) mediated by the protein kinase-C signal transduction pathway;

(6) associated with expression or down regulation of known cell proliferation and differentiation associated transcription factors such as AP-1 and Sp1.

CHAPTER 2

REVIEW OF LITERATURE

Retinoids

Retinoids are a group of naturally occurring and synthetic compounds. Retinol (vitamin A), its more active biologic derivative retinoic acid (RA), and other natural and synthetic derivatives of retinol are known as retinoids. A 'retinoid' is any substance which can elicit a specific biological response, by binding to and activating specific receptors, for which the classical ligands are retinol and/or retinoic acid (Sporn and Roberts, 1985). Retinoids share structural and functional similarity with vitamin A. Vitamin A was isolated at the start of the twentieth century as a fat soluble substance with growth promoting activity (McCollum and Davis, 1913). Its structure was subsequently determined by Karrer et al. (1931) and was found to correspond to all-*trans*-retinol (Figure 1). Following its discovery, the biochemistry and pharmacology of vitamin A have been studied extensively. The antitumor effect of vitamin A was first studied by Bollag and his group. Because of the limitations imposed by hypervitaminosis A syndrome, Bollag et al started to modify vitamin A with the aim of constructing derivatives with more specific efficacy and a wider therapeutic margin. Since then, over 1500 compounds, that are closely related to vitamin A, have been synthesized and biologically tested (Bollag, 1983).

Such compounds are categorized into three groups:

1. First generation compounds, which comprise retinol (Vitamin A) and its metabolic derivatives such as retinaldehyde, all-*trans*-retinoic acid and 13-*cis*-retinoic acid (Figure 1).
2. Second generation retinoids, which are synthetic analogues where one portion of the molecule has been altered, to produce compounds that show only part of the pharmacological profile of vitamin A. The principal compounds in this group are

etretinate (Ro 10-9359) and acitretin (Ro 10-1670) which are currently undergoing clinical evaluation.

3. Third generation compounds, where the basic molecule is extensively modified with the aim of selecting out just one of the biological activities of vitamin A, thus producing a drug that has the desired activity with minimal side-effects. To date this group of compounds is still very much at the experimental stage and no such products are yet available commercially.

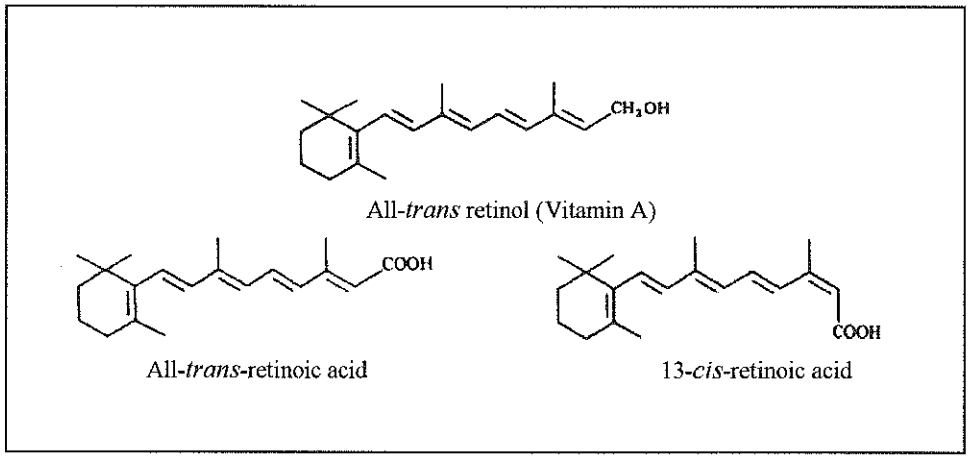

Figure 1. 1st generation retinoic acids

Retinol is a true vitamin in the sense that it cannot be synthesized by mammals therefore has to be obtained through the diet. During the digestive process any retinol present is released from proteins by the acidity in the stomach or by the action of proteolytic enzymes (Weber, 1981; Goodman and Blaner, 1984; Silvermann *et al*, 1987). The majority of the retinol released, however, is not absorbed by the normal mechanism of passive diffusion into the general circulation. Instead, a special absorption mechanism has evolved with transport via the lymphatic system. This process first involves emulsification within the lumen of the small intestine, through interaction with bile acids and pancreatic enzymes. After passage through the intestinal cell wall, retinol is esterified with long chain

fatty acids such as palmitic acid, and its absorption into the lymphatic system is then mediated via the chylomicrons. After release from this transport system retinol is then stored in the liver (Blomstrand and Werner, 1968).

The first generation retinoids consist of mainly the natural metabolites of vitamin A. These groups differ from the parent compound due to the number of chemical manipulations in the polar end group polyene side chain of the molecule resulting in various alcohols, retinoyl esters, amides, aldehydes, and amines. A large number of isomers were also synthesized from the first generation retinoids. The first derivative of vitamin A to be tested in the papilloma model was all-*trans*-retinoic acid and was found to produce a profound therapeutic effect. Similarly, 13-*cis*-retinoic acid (isotretinoin) with a superior therapeutic index compared to all-*trans*-retinoic acid, gained greatest experimental and clinical importance. It was highly effective in the treatment of cystic acne, rosacea, gram-negative folliculitis, seborrhea, and keratinizing dermatosis as well as in certain oncologic diseases such as tumors with epithelial surfaces, skin cancers and mycosis fungoides. Therapy with all-*trans*-retinoic acid has limitations because of the adverse toxicology at therapeutic doses. From the early stages it was recognized that, if synthetic retinoids were to achieve therapeutic utility, then there was a need to improve their therapeutic index. In order to achieve this goal Bollag selected and tried to predict the usefulness of a retinoid compound based on a ratio of the dose causing an antipapilloma effect and hypervitaminosis A syndrome (Bollag, 1974). Table 1 shows a comparison of the therapeutic indices of clinically important retinoids identified to date. Etretinate and 13-*cis*-retinoic acid were selected from 1500 synthetic retinoids that were evaluated. The most important feature of 13-*cis*-retinoic acid and etretinate is that they have superior therapeutic indices compared to all-*trans*-retinoic acid with etretinate showing a 10-fold improvement over the natural vitamin.

TABLE 1.

THERAPEUTIC INDEX OF CLINICALLY IMPORTANT RETINOIDS

Retinoids	Hypervitaminosis A (mg/kg)	Antipapilloma (mg/kg)	Therapeutic index
All-*trans*-retinoic acid	80	400	0.2
13-*cis*-retinoic acid	400	800	0.5
Etretinate	50	25	2.0

Effects of retinoids on normal and tumor cell differentiation

Retinoids can modulate the growth and differentiation of normal, premalignant, and malignant epithelial and mesenchymal cells *in vitro* and *in vivo*. (Lotan, 1980; Roberts and Sporn, 1984; Sherman, 1986; Amos and Lotan, 1990). RA has a role as a morphogen in pattern formation in the three-dimensional spatial organization of the different types of cells in the developing embryo (Giguere et al., 1987). The physiologic effects of retinoids *in vivo* include: prevention of squamous cell differentiation in nonkeratinizing epithelial tissues; control of differentiation of certain mesenchymal tissues, and regulation of certain aspects of the immune response (Lotan, 1980; Sherman, 1986; Dennert, 1984).

Retinoids exert distinct effects on the growth and differentiation of various types of tumor cells *in vitro* (Lotan, 1980; Amos and Lotan, 1990; Lotan et al., 1981; Roberts and Sporn, 1984) and *in vivo* (Halter et al., 1988; Lippman et al., 1989). They enhance differentiation of embryonal carcinoma cells along endodermal, neuronal, or mesenchymal pathways, induce neuronal differentiation in neuroblastoma cells, myeloid differentiation in HL-60 promyelocytic leukemia cells, monocytoid differentiation in premonocytic leukemia cells, erythroid differentiation in erythroleukemia cells, and melanocytic differentiation in

melanoma cells (Roberts and Sporn, 1984; Amos and Lotan, 1990). Other important features of retinoids are their ability to suppress carcinogenesis and inhibit tumor growth in experimental animals, (Lotan, 1980; Roberts and Sporn, 1984; Sherman, 1986; Amos and Lotan, 1990) and their efficacy in chemoprevention and therapy of certain types of malignancy in humans. (Lippman et al., 1989; Castaigne et al., 1990). Because of these properties, retinoids are considered one of the more promising candidates for the non toxic approach to cancer prevention and therapy, known as 'differentiation therapy' (Lotan et al., 1990; Reiss et al., 1986). The basis for this approach is to suppress or reverse the malignant phenotype induction of differentiation or by restoration of responses to normal growth control mechanism in cancer cells (Lotan et al., 1990; Reiss et al., 1986).

Effects of retinoids on the growth of cultured tumor cells *in vitro*

RA and other retinoids inhibit the growth of a large number of murine and human tumor cells in monolayer cultures and in agar or agarose semisolid media (Amos and Lotan, 1990). Among the cells inhibited by RA are B16 melanoma, (Lotan, 1986; Amos et al., 1989) human breast carcinoma, (Halter et al., 1988) mouse mammary carcinoma, (Couch et al., 1987) human lung squamous carcinoma, (Olsson et al., 1985) mouse squamous carcinomas, (Clifford et al., 1989) and mouse fibrosarcoma. In most cell types the inhibition was time-dependent (detectable within 2-3 days), dose-dependent in the range between 10^{-10} and 10^{-5} M, and reversible. The effects were cytostatic and cells accumulated in the G_1 phase of the cell cycle (Lotan et al., 1981; Olsson et al., 1985). However, the effect of RA on the differentiation of cancer cells is not well documented. Furthermore the growth inhibition and the subsequent differentiation of colon cancer cells by RA remains to be elucidated.

The role of host immune responses in the effect of retinoids on metastasis

The development and growth of a primary tumor often requires that the tumor cells escape the immune surveillance mechanisms of the host. In addition, metastatic cells have to survive in the circulation, where immune cells bind at the secondary site, where the establishment of a metastasis from a few arrested cells depends on their ability to withstand the assault by the host's humoral response and infiltrating- cytotoxic cells.

Several studies have shown that retinoids can affect host antitumor immune response directly by enhancing T cell-mediated antitumor responses as well as increase the activity of other arms of the immune response (Dennert, 1984). Retinoids also augmented host response against melanoma cells by acting on the tumor cells themselves to increase the expression of antigens (Zelent et al., 1989). In addition, retinoids decreased the shedding of tumor cell plasma membrane-fragment, which suppress the activation of T lymphocytes and macrophages and abrogate antitumor immune response (Jiang et al., 1990). Furthermore, in some models of experimental metastasis of mammary carcinomas, squamous carcinomas, and fibrosarcomas in syngeneic mice, it has been shown that the suppression of metastases formation by retinoids is the result of enhancement of host immune responses, because only immunogenic tumor cells responded to treatment with the retinoid trimethymethoxyphenyl analogue of ethyl retinoate, whereas nonimmunogenic tumor cells were not inhibited from growing locally and forming metastases (Eccles et al., 1985). A subsequent investigation established that the inhibitory effects of the retinoid on tumor growth and metastasis depended on T lymphocytes because no tumor suppression was observed in mice congenitally deficient in T lymphocytes (nude mice), or rendered deficient by thymectomy plus irradiation, or by treatment with cyclosporin (Eccles et al., 1985).

Studies on the mechanism(s) of retinoid action

Many different theories concerning the mechanism of action of retinoids were developed over the past 30 years. For example, since retinoids are lipophilic, some researchers suggested that retinoids might damage the plasma membranes of cells, leading to cytotoxicity. This is almost certainly not true, at least for the low RA concentrations (10^{-9} to 10^{-5} M) that lead to biologic effects. These effects of RA are presumably mediated by intracellular receptors.

The first high affinity RA binding protein to be identified was the cellular RA binding protein (CRABP) (Chytil and Ong, 1979), a small cytoplasmic protein (approximately 15 kD) with no obvious DNA binding motif. Despite much effort, the role of the CRABP in RA action is not understood. Some researchers currently believe that the CRABP facilitates the movement of RA to the nucleus, perhaps acting as a shuttle protein, whereas others think that the CRABP may sequester RA, preventing RA from moving to the nucleus and thereby decreasing the cellular response to RA.

A second group of high affinity retinoic acid receptors (RARs) has also recently been identified; these proteins exhibit some sequence homologies with the protein receptors for steroid hormones, thyroid hormone, and vitamin D3, which suggests that these newly identified receptors are RA-dependent trans-acting enhancer factors that regulate the transcription of RA-responsive genes. Stated slightly differently, by analogy with steroid hormone receptors, these RARs must act in the nucleus by binding to specific DNA regulatory sequences to activate specific sets of genes in the presence of RA. The first RA receptor to be cloned and sequenced was RARα (Petkovich et al., 1987). Two other RARs have since been identified, RARβ and RARγ (Zelent et al., 1989). Interestingly, although all three known RA receptors are related in sequence, comparison of the human and mouse RAR amino acid sequences reveals that the interspecies conservation (i.e., human RARα and mouse RARα, and so forth) is higher than the

conservation of all three receptors within one species. It is likely that the RARs themselves are transcription factors that regulate the genes that encode for several other different types of transcription factors. In fact, exciting work within the past 3 years has shown that the genes for several transcription factors are primary targets of RA (de The et al., 1990). An "RA primary target" gene is one type of RA-responsive gene; such a gene can be transcriptionally activated or inhibited in a rapid and protein synthesis-independent fashion after the addition of RA to a cell. The fact that some of the "RA primary target" genes are transcription factors adds further complexity, as these factors can subsequently regulate the expression of a variety of other specific cellular genes. Although many RA-responsive genes have been described in a variety of different RA-responsive cell types, retinoic acid response elements (RAREs) (i.e., portions of promoter DNA that are involved in the RA response of the gene) have thus far been identified in the 5' flanking region of the murine laminin BI gene, a gene encoding a subunit of the extracellular matrix protein laminin.

Human colon cancer as a model for the study of the effects of retinoids on tumor cells

Colon cancer is one of the most common malignancies in the United States. It is the third most common human malignancy and it occurs in 5% of men and 6% of women. Each year approximately 140,000 new cases are diagnosed and there are 60,000 deaths attributed to the disease. In addition, the incidence and survival rates have not improved (Sugarbaker et al., 1985; Schwartz, 1990). It is thought to develop from a sequential step of progressive alterations (Figure 2) of normal colonic mucosal cells through a polyp (adenoma phase) before becoming malignant (Cho and Vogelstein, 1992).

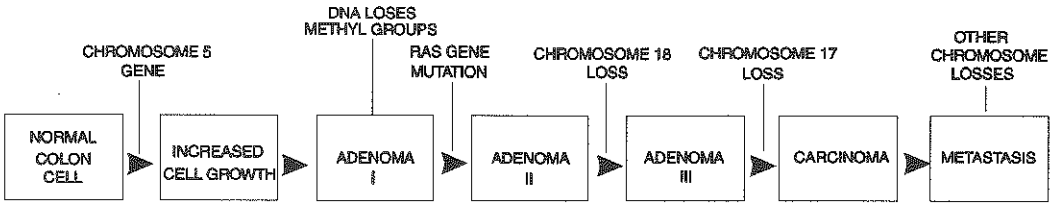

Figure 2. Multistep process in the formation of colon carcinoma.

There are two inherited forms of colon cancer that have been well-studied and are thought to have different molecular etiologies. Although these inherited predispositions are relatively rare, it is thought that the genes responsible play major roles in the common sporadic (non-inherited) form of the disease. The form of inherited susceptibility to colon cancer, about which the most information exists is familial adenomatous polyposis (FAP). The incidence of FAP is estimated at about 1 in 7,000 (Reed and Neel, 1955; Pierce, 1968), but the gene responsible (FAP gene) is estimated to be involved in >40% of sporadic colon cancer (Solomon et al., 1987). FAP is inherited as an autosomal dominant trait with high penetrance and is characterized by the presence in the colon of numerous adenomatous polyps by the age of twenty. Unless colectomy is performed, death from carcinoma approaches 100% by age 55 (Sugarbaker et al., 1985). In 1973, Knudson postulated that the disease might involve two genetic events, one of which is inherited and causes hyperplasia and polyp formation, and the other of which occurs in one of the polyp cells to produce a carcinoma (Knudson, 1973). He reasoned that the sporadic form of colon cancer might involve the same two events, but they would both take place somatically. He further proposed a simple model in which the two genetic events take place in the two alleles of the same genetic locus. Tumor formation in both the sporadic and inherited forms would result from loss of function of both alleles of a tumor suppressor gene or anti-oncogene, a gene whose function is anti-oncogenic.

Current molecular evidence is consistent with the Knudson anti-oncogene model. The FAP gene has recently been mapped to chromosome 5q (Bodmer et al., 1987; Leppert et al., 1987). Carcinomas arising in FAP individuals, as well as those that arise in individuals without familial polyposis, frequently show allele loss on chromosome 4, and chromosome 5q (Solomon et al., 1987; Okamoto et al., 1988). This finding is consistent with Knudson's two-event hypothesis if one assumes that the FAP gene behaves as a tumor suppressor gene or anti-oncogene. Allele loss on 5q is evidence of inactivation of one allele of the FAP locus by a global event such as chromosome deletion, chromosome loss, or genetic recombination. Function of the other allele is presumably lost through a local lesion such as point mutation or a small deletion or insertion. For FAP individuals, one lesion is inherited, and the other occurs somatically. For sporadic cases, both occur somatically. Allele loss on 5q has also been documented in 29% of polyps occurring in non-polyposis patients (Vogelstein et al., 1988). This finding is consistent with the hypothesis that loss of function of one FAP allele leads to polyp formation. In those polyps where allele loss on 5q was not observed, one could speculate that loss of function of one FAP allele may have occurred through a local lesion. Because the polyps of FAP patients do not show allele loss at 5q, it is presumed that the inheritance of one defective FAP allele is sufficient to induce polyposis. Allele loss on 5q is generally seen in only 20% (Solomon et al., 1987) to 40% of carcinomas (Solomon et al., 1987; Okamoto et al., 1988) so 20-40% of cases involve loss of FAP gene function through a global event. However, those cases in which both alleles are lost through a local lesion are not scored in these experiments. From studies on the retinoblastoma gene, it is estimated that such cases may constitute 50% of the total (Dryja et al., 1984). Recently another gene has been mapped to chromosome 5q21 which was found closely linked to FAP gene (Kinzler et al., 1991). The new gene is called "mutated in colorectal cancers" (MCC) and has been detected in sporadic colon cancers (Nishisho et al., 1991). It is estimated that MCC and

FAP gene loci are involved in initiation of a major fraction (>40%) of sporadic colon cancer (Kinzler et al., 1991).

In addition, other chromosomal regions show loss of heterozygosity in colon carcinomas and may contain additional anti-oncogene loci. For example, such loss is seen for chromosomes 17p and 18q15 (Monpezat et al., 1988) which were considered as putative tumor suppressor genes.

Oncogenes and colon cancer

Mutations in the cellular genome affecting the expression or function of genes controlling cell growth and differentiation are considered to be the main cause of colon cancer. The mutation or overexpression of protooncogenes are well characterized in the progression of colon carcinoma (Cho and Vogelstein, 1992). Oncogenes were first identified as viral genes responsible for the ability of oncogenic retrovirus to produce tumors in susceptible hosts. It was later demonstrated that the genomes of all vertebrates contain homologues of the retroviral oncogenes. These cellular genes are termed protooncogenes (Bishop, 1983). The protooncogenes differ from their viral counterparts in that they must be activated by point mutation or by changes in timing and amount of expression to become oncogenic. Much effort has been invested in understanding the role of protooncogenes in normal cell functions (Bishop, 1985; Weinberg, 1985), as well as their involvement in cell transformation and tumorigenesis (Bishop, 1987). It is generally presumed that the protooncogenes play essential roles in the coordination and regulation of cell proliferation, functioning to expand pools of immature precursor cells in development of tissues and organs and support continuous cell division in tissue renewal, such as the intestinal and colonic epithelium and the hematopoietic system. Changes in the activity of such genes due to mutation or the failure to properly regulate their expression can lead to transformation and tumorigenesis.

The levels at which protooncogenes act to regulate and oncogenes act to deregulate cell proliferation can be separated into signal reception, transduction, mediation, and response. These events result in transmission of signals generated by ligand interactions with specific cell surface receptors into changes in intracellular metabolism and eventually into initiation of the process of cell division (Gilman, 1987). The level of signal reception is represented by membrane receptors for growth factors and the factors themselves, e.g., the epidermal growth factor receptor (homologous to the *erb* B oncogene) and platelet derived growth factor (homologous to the *sis* oncogene) (Yamamoto et al., 1986; Deuel et al., 1985). The signal transducing protooncogenes include *src* and the *ras* gene family (Willingham et al., 1983). These genes are presumed to play a central role in transduction of signals into the cell nucleus. Transcription regulators like the *fos* and *jun* protooncogenes would be considered mediators of the transduced signals (Moore et al., 1988). This category may also include the retinoblastoma gene, an anti-oncogene. These mediators are presumed to induce expression of target genes, such as members of the *myc* protooncogene family (Filmus et al., 1987). Expression of these target genes is considered essential for replication of the DNA and for cell proliferation. The process of tumor formation involves uncoupling of the process of signal transduction from its normal control mechanisms. The following are some examples of how such uncoupling can occur: a growth factor receptor may become altered so that it no longer requires binding to growth factor to be active; thus, it would continually transmit the signal to proliferate in the absence of growth factors. The signal transducing oncogenes *src* and *ras* can be activated by point mutation so that they no longer need to interact with the receptor system to send signals to the nucleus. Target genes, such as c-*myc* gene. can become deregulated so that they produce their protein product continuously and are no longer responsive to the signaling system. In model systems, such as rat embryo fibroblasts, deregulation must occur at two levels in order to transform a normal cell into a tumor cell (Land et al., 1983). For example, tumorigenic

cells can be produced if *ras* is activated by point mutation, and c-*myc* is deregulated to give constitutive high level expression. It is not known whether carcinogenesis in the colon requires deregulation at multiple levels or, if it does, how many events may be involved. However, recent evidence indicates that both the *myc* and *ras* oncogenes may play a role in colon cancer (Yokota et al., 1986; Pories et al., 1992).

The c-*myc* protooncogene was originally identified as the cellular homologue of the transforming gene of the avian retrovirus MC29 (myelocytomatosis virus no. 29). which causes myelocytomatosis, sarcoma, and carcinoma in inoculated birds (Beard et al., 1975). The *myc* gene family currently contains 6 mammalian members: (DePinho et al., 1987) c-*myc*, L-*myc*, N-*myc*, R-*myc*, P-*myc*, and B-*myc*, all of which are related by stretches of amino acids, the so-called *myc* box. N-*myc* appears to be involved in tumor progression in neuroblastoma (Brodeur et al., 1984), whereas c-*myc*, N-*myc*, and L-*myc* have all been implicated in tumor progression in small cell carcinoma (Brooks et al., 1987). c-*myc* is the only member implicated in colon carcinogenesis.

The c-*myc* gene (Cole, 1986), located on human chromosome 8q24, consists of three transcribed exons (regions expressed as RNA) spanning about 6 kilobases; the second and third exons are translated into a 439 amino acid protein with a predicted molecular weight of about 49 kilodaltons. Immune antiserum precipitates a phosphorylated nuclear protein of 62 to 67 kilodaltons relative molecular weight that has a short halflife (about 30 minutes) and binds single and double stranded DNA *in vitro* (Persson and Leder, 1984). It is generally believed that the *c-myc* protein product is essential for cell proliferation. C-*myc* expression goes up in response to mitogenic signals in cells that have been growth-arrested and is associated with a state of competence to progress into S phase (Kelly et al., 1983). Although it is clear that *c-myc* expression is intimately linked to entry and exit from the cell cycle, its exact role in cell proliferation remains unclear. C-*myc* protein is reportedly associated with the nuclear matrix (Eisenman et al., 1985) and has been postulated to play an active role in DNA replication

(Gutierrez, 1988). Possibly by binding to regions on the DNA that serve as initiation sites for DNA synthesis. Thus, it may affect the processing or half-life of other gene products that are essential for cell proliferation.

In tumors, *myc* activation appears to result from deregulation of the level and timing of expression, rather than from mutations in the protein product. Constitutive, elevated expression of the RNA and protein has been implicated as an initiating event in oncogenesis in a wide variety of tumor types, including colon carcinoma. The first documentation of *c-myc* deregulation in a tumor was the demonstration that, in avian leukosis virus induced bursal lymphomas, the chicken c-*myc* gene was activated by promoter insertion (Hayward, 1981).

In 60% to 80% of colorectal adenocarcinomas, *c-myc* RNA has been shown to be expressed at levels 5 to 40 times those in normal colonic mucosa, but the gene is not amplified or detectably rearranged (Erisman et al., 1985). Subsequent studies confirmed the deregulation of expression at both the RNA and protein levels in primary tumors, as well as in colon carcinoma cell lines (Rothberg et al., 1985).

Another family of genes that is frequently found to harbor a mutation in human colon tumors is that of the *ras* gene. The *ras* family of protooncogenes (Barbacid, 1987; Lacal and Tronick, 1988) originally identified as the cellular homologues of the transforming agents of the Harvey and Kirsten murine sarcoma viruses (Lacal and Tronick 1988). In humans, there are three members of the *ras* gene family: Ha-*ras,* Ki-*ras, and* N-*ras.* Each codes for a structurally related 21,000 dalton protein called p21, consisting of 189 amino acids (for Ha and N-*ras)* or 190 amino acids (for Ki-*ras*). A cysteine residue at position 186 gets posttranscriptionally palmitolylated, allowing *ras* proteins to associate with the inner surface of the cell membrane (Furth et al., 1982). *Ras* proteins also bind guanine nucleotides and hydrolyze GTP to GDP (Sweet et al., 1984). The properties of membrane association and GTPase activity implicate *ras* proteins as putative signal transducers, involved in the transmission of signals generated by ligand interaction

(Gilman, 1987) with specific cell surface receptors into changes in intracellular metabolism. The first transforming genes to be isolated from human tumors were activated counterparts of members of the *ras* gene family (Der et al., 1982). It was later determined that activation involved point mutation in the protein coding sequences of the gene (Reddy et al., 1982). Thus, a single amino acid change could produce a transforming protein. Positions most commonly altered were glycine at codon 12 and glutamine at codon 61 (Barbacid, 1987). Mutations altering these codons decrease the rate of GTP hydrolysis. Thus, it was postulated that the p21-GTP complex represented an activated complex that could stimulate cell division. The exact mechanism by which such stimulation occurs remains to be determined.

Ras alleles that have been activated through point mutation are commonly found in colon carcinoma cell lines and in primary tumors (McCoy et al., 1984). Survey of human colorectal tumors revealed that 50% of the primary colon carcinomas appear to express increased immunoreactivity of the of the p21 product of the *ras* gene. (Gallick et al., 1985). Biopsy specimens contained *ras* alleles with an activating mutation (Bos et al., 1987).

Carcinoembryonic antigen and colon cancer

Carcinoembryonic antigen (CEA), a widely used human tumor marker, is expressed in most of the human carcinomas, especially gastrointestinal, lung, and breast carcinomas (Schwartz 1990). Its presence in these tumors has led to its use as a marker in several modalities of human carcinoma management (Fuks et al., 1974). CEA is a highly glycosylated glycoprotein with a molecular mass of 180,000 and consists of a single polypeptide chain (protein, 72,800) (Beauchemin et al., 1987). It is a member of a family of structurally related genes for which the complexity of expression varies in a tissue-specific manner. (Thompson and Zimmermann, 1988). The normal function of CEA, its role in carcinogenesis, is still under investigation. Producing CEA is one of the properties

of human colon cancer cells. In general, the tissue and serum levels of CEA in colon cancer patients reflect the differentiation of the tumor, with higher CEA contents being characteristics of the well differentiated tumors, while poorly differentiated or anaplastic tumors have low or no CEA content (Khoo et al., 1973). The control of expression of genes of the carcinoembryonic antigen family was investigated in malignant (adenocarcinomas) and nonmalignant human colonic tissues. The tumor/normal ratios of immunoreactive CEA were reported from 2- to greater than 100-fold. Furthermore, there was no direct proportionality between mRNA levels and gene product expression observed, suggesting that the variations in CEA expression in human colonic tissues result from both transcriptional and posttranscriptional control mechanisms (Boucher et al., 1989). Tumor-differentiation-related expression of the CEA have been shown to be modulated by retinoids in several model systems (Shiveley and Beatty, 1985). In a study, RA was reported to induce CEA production in one colorectal cell line (DLD-2), among two others (clone-D, MIP-101) while inhibiting the growth markedly in all three. (Niles et al., 1988).

HLA class I antigens

The major histocompatibility complex (MHC), referred to as HLA in humans, encodes three classes of gene products: class I contains major determinants of graft rejection and serve as self-recognition elements for cytotoxic T-cells; class II elements function in antigen presentation and in self-recognition by helper T-cells; class III are components of the complement system (Klein, 1986). The products of class I histocompatibility antigens are 45-kDa polymorphic cell-surface glycoproteins, present on most somatic cells in association with β_2-microglobulin (Ploegh *et al.*, 1981). The development of malignant tumors represents not only neoplastic transformation but also the failure of host resistance to eliminate aberrant cells. Because neoplastic cells frequently express surface antigens (neoantigens), they are recognized as foreign and

eliminated by the host. Effective immunosurveillance requires not only an immunocompetent host but also expression of MHC class I antigens along with tumor neoantigens. Altered expression of MHC antigens has been proposed as a mechanism which protects tumor cells from immunosurveillance (Tanaka et al., 1986). The basic idea concerning the role of class I molecules in oncogenesis is that, since they are required in the presentation of neoantigens on tumor cells to the cytotoxic T-lymphocytes, their absence from the cell surface may lead to escape of these tumors from immunosurveillance. Experimental evidence supports this concept (Tanaka et al., 1985). Reduced expression of class I HLA antigens has been observed for several tumor types (Brocker *et al.*, 1985; Garrido, 1986). It has been suggested that a reduction in the amount of class I HLA might be associated with increased malignancy of tumor cells (Brocker *et al.*, 1985; Garrido, 1986). For example, transformation of cells by adenovirus type 12 is associated with reduced expression of MHC class I antigens and increased tumorigenicity in syngeneic immunocompetent hosts. Expression of transfected MHC class I genes in the adenovirus type 12-transformed cells can abrogate their tumorigenicity. When the expression of HLA class I and II antigens was studied in specimens of human colon cancer expression of HLA class I antigen expression was found to be dramatically reduced (undetectable in 28%, diminished in 68%, normal in 4%) compared to control (McDougall et al., 1990).

Ornithine decarboxylase and colon cancer

Ornithine decarboxylase (ODC) catalyzes the first step of polyamines' (putrescine, spermidine and spermine) production from L-ornithine. The direct mechanism of action of polyamines remains unclear, but they are required for DNA synthesis and are essential for cell growth (Berdinskikh et al., 1991; Elitsur et al., 1992). ODC is a key enzyme in cell proliferation through its involvement in the biosynthesis of polyamines. Polyamines are required for the synthesis of nucleic acids and cell replication (Denhart et al., 1986). ODC

has some unique properties: it is induced by a variety of stimuli; has a very short half life; and is stimulated by hepatectomy, hormones, growth factors, and tumor promoters, such as phorbol esters. Several studies have reported elevated levels of ODC activity in neoplastic human colon versus normal-appearing colonic mucosa (Guillem et al., 1990; Elitsur et al., 1992). and higher levels of ODC activity have been reported in dysplastic polyps than in nondysplastic polyps (Luk and Baylin, 1984). In non familial colon adenocarcinomas, ODC activity has been consistently found to be elevated, compared to adjacent mucosa. Moreover, it has been previously shown that ODC activity correlates with the degree of colonic dysplasia in patients with familial polyposis (Herrera-Ornelas et al., 1987). In experimental models of colon carcinogenesis, a colon carcinogen (azomethane) increased ODC activity within 4 hour after a single dose was used (Luk et al., 1986). In another study an ornithine analog, 2-difluoromethylornithine (DFMO), reduced the incidence of colon cancer in mice induced by 1,2-dimethylhydrazine (DMH) (Kingsnorth et al., 1983). This suggests that enhanced ODC activity may have an important role in colon tumor development. Because of its role as key regulatory enzyme in the pathway of polyamine biosynthesis, it is suggested that over-expression of ODC and consequent elevated rates of polyamine synthesis may provide a selective growth advantage for colonic neoplastic cells (Hietala et al., 1990). Although studies have been conducted on the effects of regulatory agents on ODC expression, the effect of retinoids on the regulation of ODC expression unknown and remains to be investigated.

Protein kinase C and colon cancer

The calcium- and phospholipid-dependent protein kinase (protein kinase C, PKC) is a family of closely related isoenzymes which phosphorylates proteins on serine and threonine residues (Nishizuka, 1988). Many hormones, growth factors, and neurotransmitters bind to cell surface receptors and activate phospholipase C, which in turn hydrolyzes phosphotidylinositol biophosphate into inositol triphosphate (IP_3) and

diacylglycerol (DAG) (Nishizuka, 1986). IP_3 induces an intracellular release of calcium, whereas DAG binds and activates PKC (Nishizuka, 1986). It is known that PKC can also act as a receptor for phorbol ester tumor promoters (Persons, 1988). Therefore, PKC has a role in a multitude of cellular functions such as growth, differentiation, and tumorigenesis (Nishizuka, 1986; Sposi NM, 1989). The critical substrates for phosphorylation by PKC that result in cell proliferation have not been conclusively identified but they include growth factor receptors and extranuclear and nuclear protooncogene products (Nishizuka, 1986). PKC is known to be associated with increased proliferation in several cell types including colonic epithelial cells (Sakanoue et al., 1991; Craven and DeRubertis, 1987). It has been reported that the regulation of cell division by growth factors, as well as cell transformation induced by several viral oncogenes, involves the activation of protein kinases (Sposi NM, 1989). When human promyelocytic leukemia cell line HL-60 cells were treated with chemical reagents such as RA, dibutyryl cAMP, dimethylsulfoxide, and nicotinamide, the cells were induced to differentiate into morphologically and functionally mature myelocytes (Hashimoto et al., 1990; Tanaka et al., 1992). Molecular cloning and biochemical characterization of this enzyme have indicated the presence of seven isoforms of which best characterized are the α, β (I/II), and γ in mammalian tissues (Ohno et al., 1991). RA-induced PKC isoforms are differently expressed among and within tissue types and tumors. In the RA-induced morphological differentiation of human neuroblastoma cells a decrease of PKC-α gene expression along with a down regulation in N-*myc* mRNA was observed (Tonini et al., 1991). RA-induced growth arrest and differentiation in B16 mouse melanoma cells were accompanied by an increase in the PKC-α and -β mRNA expression, but no -γ mRNA expression (Rosenbaum and Niles, 1992). In HL-60 cells several differentiation inducers including RA stimulated PKC-α and β isoform activities. Further, among these inducers only RA dramatically enhances the activity of an unidentified PKC isoform (Tanaka et al., 1992). The evidence of *in vitro* studies supports the possibility that activation and/or

translocation of PKC from cytosolic to particulate fraction may serve as a positive intracellular signal to colonic epithelial cell growth in retinoid-mediated induced differentiation (Michaeli et al., 1992).

AP 1 and Sp 1 Transcription Factors

Sequence-specific DNA binding proteins are believed to play a key role in such vital cellular processes as transcription, replication and recombination. Cells react to environmental and developmental stimuli by changes in the specificity and efficiency of transcriptional regulation. In transcription, these proteins regulate the rate of mRNA synthesis by binding to discrete DNA sequences termed enhancers or promoters. The activity of these transcription factors may be regulated in a cell-specific or cell-cycle dependent manner as well as responding to extracellular signaling.

The AP-1 transcription factor was first identified as a protein which bound specifically to the palindromic DNA sequence TGACTCA and variations of this motif in the enhancer region of the SV40 T antigen and human metallothionine genes (Lenardo and Baltimore, 1989). AP-1 *cis* enhancer sequences are now thought to be involved in the control of expression of a variety of genes inducible by phorbol esters (Cross et al., 1989). A major AP-1 binding protein has been identified as the c-*jun* protooncogene (Muesing et al., 1987). In addition to c-*jun*, AP-1 is constituted by the product of another protooncogene, c-*fos*. The *Fos* protein forms a non-covalent association with the *Jun* oncoprotein via leucine zipper dimerization domain. Ap-1 is constituted by a mixture of products from the various genes that belong to the *fos* and *jun* families. Although *fos* and *jun* contain functional domains contributing to DNA binding only *Jun* is able to form homodimers. *Fos* on the other hand, when associated with *Jun*, appears to enhance dramatically the binding affinity of the heterodimer.

Components of the AP-1 transcription factor are involved as 'third messenger' in signal transduction pathways, establishing the final link through which extracellular cues

can mediate their effect on gene transcription. Changes in the cellular environment modulate the DNA-binding and hence also the transcriptional activity of the members of AP-1 family. These phenomena are likely to occur via effects on various signal transduction pathways such as the PKC or protein kinase A (PKA) pathways. The alterations in AP-1 activity can be caused either by a transcriptional induction of the *fos* or *jun* gene families or by posttranslational modifications of both oncoproteins or their regulatory factors. RA has been shown to act as a negative regulator of AP-1-responsive genes. This regulation occurs via the formation of a nonproductive complex between RAR and c-*jun* (Schule et al., 1991).

Transcription factor Sp1 is a protein present in mammalian cells that binds to GC-rich polynucleotide sequence elements (GC box) and selectively activates mRNA synthesis from genes that contain functional recognition sites (Dynan and Tjian, 1983). The biochemical analysis of cellular trans-activators involved in promoter recognition provides an important step toward understanding the mechanisms of gene expression in animal cells. The promoter selective transcription factor, Sp1, has been purified from human cells by sequence-specific DNA affinity chromatography. Isolation and renaturation of proteins purified from sodium dodecyl sulfate polyacrylamide gels allowed the identification of two polypeptides (105 and 95 kilodaltons) as those responsible for recognizing and interacting specifically with the GC-box promoter elements characteristic of Sp1 binding sites (Briggs et al., 1986). The DNA binding activity of Sp1 was localized by expression of truncated fragments of Sp1 in E. coli. In this region, Sp1 has three contiguous Zn(II) finger motifs, which are believed to be metalloprotein structures that interact with DNA and via which Sp1 interacts with DNA. (Kadonaga et al., 1987; Kadonaga et al., 1988). In SV40 virus, it was determined that transcriptional activation by Sp 1 requires sequences within tandem 21 bp repeats located 70-110 bp upstream of the transcription initiation sites. Results have indicated that direct binding of Sp 1 to sequences in the upstream promoter element is the mechanism by which this factor activates transcription by RNA polymerase II at the SV40

early promoter (Dynan et al., 1985). In a study of Sp1 binding sites in protooncogenes the promoter region of the human c-Ha-*ras*1 has been shown to initiate RNA transcription at multiple sites and contain repeated copies of the hexanucleotide GGGCGG and its inverted complement CCGCCC, referred to as GC boxes (Ishii et al., 1986). These GC boxes consist of sequences identical to those found in the SV40 early promoter, where the human cellular transcriptional factor Sp1 binds. Footprinting analysis with deoxyribonuclease was used to show that Sp1 binds to six GC box sequences within the c-Ha-*ras*1 promoter. An *in vivo* transfection assay has shown competition between the 21-base pair repeats of the SV40 promoter and the c-Ha-*ras*1 promoter for common regulatory factors.

CHAPTER 3

MATERIALS AND METHODS

Culture conditions of human colon adenocarcinoma cell line

HT-29 human colon carcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). This cell line was used because of its well-documented tumorigenic potential (Trainer et al., 1988). All cell culture media and supplements were obtained from Gibco BRL (Grand Island, NY). Cells were propagated in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air and handled in sterile conditions. Medium was changed once a week and cultures were passed every week. To detach the cells from the flask, for passing and/or harvesting, trypsin-EDTA solution (0.025% trypsin: 0.02% EDTA v/v final concentration) in phosphate buffer saline without calcium and magnesium (PBS) was used and trypsinization was stopped by adding serum containing medium to the culture flasks.

Retinoid Treatment of the Cells:

Retinol, all *trans* RA, and 13-*cis* RA was obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of retinoids were prepared in absolute ethanol at a concentration of 10^{-2} M and were kept at -80°C in dark until their use. All phases of the procedure involving the addition of the RA(s) to the culture flasks were held under the illumination of subdued yellow light. Where older than two weeks, stock of RA was not used and a new batch of stock solution was prepared.

HT-29 cells at their log-phase of growth were collected and 20,000 cells/cm^2 of a flask were seeded into cell culture flasks in the above-mentioned media. Cell numbers were measured by counting cell suspensions with a hemocytometer and viability was assessed by exclusion of 0.2% trypan blue. Cells were allowed to attach to the flask for 24 hours. Then, they were treated with retinoids that were prepared in absolute ethanol that did not exceed 0.01% (v/v) of the culture medium. The same volume of ethanol alone was added to the control cultures. Cell cultures were harvested after various incubation periods (1 to 7 days) with retinoid(s) as explained in subsequent sections.

MTT assay

Determination of the cell growth was achieved using a colorimetric assay. The technique is based on the ability of live but not dead cells to reduce a tetrazolium-based compound (dimethylthiazol-diphenyltetrazolium bromide, MTT, Sigma Chemical Co., St. Louis, MO) to a blue formazan product by the mitochondrial dehydrogenase and it provides a high correlation ($r^2 > 0.96$) between the number of live cells and optical density of the cell suspension (Carmicheal et al., 1987). Following appropriate incubation of cells with or without retinoids in 6-well tissue culture plates, 0.1 mg (50 ul of 2 mg/ml) MTT was added to each well and incubated at 37°C for a further 4 hours. The medium was then aspirated, and the blue MTT-formazan product was solubilized by addition of 2 ml dimethyl sulfoxide. The plates were shaken for 10 min., and the absorbance at 540 nm was read.

RNA isolation and Northern blot analysis

Total cellular RNA of HT-29 cells was isolated using the modification (Maniatis et al., 1982) of guanidinium thiocyanate (GuSCN)-cesium chloride (CsCl) ultracentrifugation method of Chirgwin et al. (1979). For each condition adherent cells were rinsed with PBS, and lysed in situ by the addition of 4 M GuSCN. The lysate was shredded by 10 strokes in

a Dounce homogenizer, after which the lysate was layered on a 5.7 M CsCl cushion and centrifuged for 18 hours at 100,000g at 15°C. The RNA pellet was recovered and dissolved in 10 mM Tris-HCL, pH 7.5, with 0.1 mM EDTA. After being precipitated with NaOAc and ethanol, the 260/280 ratio as a crude indication of the purity of the RNA was measured and quantitated by using the absorbance at 260 nm. Twenty ug of total RNA from the different conditions was added to denaturing buffer (2X gel running buffer, 6.5% formaldehyde, and 50% formamide). Samples were denatured at 65°C for 15 min. and chilled on ice, after which an equal volume of gel loading buffer, containing 50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromphenol blue, 0.25% xylene cyanol FF, was added. Electrophoreses of the samples were carried out at a maximum of 10V/cm of gel length in a denaturing 1.4% agarose gel containing 6.5% formaldehyde, 1X MOPS (3-(N-morpholino)-propanesulfonic acid) buffer, and 1μg/ml ethidium bromide. The electrophoreses were stopped when the bromphenol blue tracking dye has run approximately 75% of the gel length. RNA was transferred to nylon membranes by capillary blotting. To permanently fix the RNA, membranes were air dried and then baked in vacuo for 2 hours at 80°C. Membranes were incubated at 42°C for an hour in prehybridization solution (0.1 ml/cm^2 of filter area containing 10% SDS, 1M NaCl, 50% deinonized formamide, 10% dextransulfate, and 100 mg/ml denatured salmon sperm DNA) in heat sealable bags. The class I HLA cDNA probe, HLA-B7 was obtained from ATCC (Rockville, MD) as the 1.4 Kb PstI insert of the recombinant plasmid pD001. c-H-*ras*, c-N-*ras* and c-Ki-*ras* probes obtained from Oncogene Science (Uniondale, NY). were 0.8 kb, 0.5 kb and 2.0 kb cDNA restriction fragments respectively. The ODC probe was obtained from ATCC (Rockville, MD) as 1.8 KB EcoRI-EcoRI fragment of a human ODC cDNA (Hickok et al., 1987).

Hybridizations were performed at 42°C for 24 hours in the prehybridization solution with the addition of 10^6 dpm/ml of denatured labeled DNA probes. HLA class I B7 and ODC probes were labeled by random primer extension (Feinberg and Vogelstein,

1983; Feinberg and Vogelstein, 1984) in the presence of dCTP [α–^{32}P] (Du Pont, Wilmington, DE) and three unlabeled dNTPs using Klenow fragment of E. coli DNA polymerase I. All three *ras* cDNA probes were end labeled with ATP [γ–^{32}P] (Du Pont, Wilmington, DE) by T4 polynucleotide kinase. After two consecutive 5 minutes washes with 2X SSC at room temperature and another two consecutive washes with 2X SSC /0.01% SDS at 55°C the membranes were washed twice with of 0.1X SSC//0.1% SDS at room temperature for 15 minutes. Blots were then exposed to X-ray films at -80°C with an intensifiying screen and developed. The intensity of the bands were quantitated by a video densitometer scanner on transmitting mode using a Bio-Rad 1D Analysis software (Bio-Rad Laboratories, Richmond, CA).

Preparation of protein samples

After treating the cells for various times with RA, cells were harvested by trypsinization and pellets of cells were homogenized using either a dounce homogenizer or a sonicator in 20 mM Tris pH 7.5, 1 mM EDTA homogenizing buffer containing proteinase inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 ug/ml leupeptin and 50 mM 2-mercaptoethanol. The lysates were then sedimented at 12,000 x g for 10 min. at 4°C. The supernatant was removed as the protein extract and stored at -80°C until use. Protein determinations were done according to Bradford using albumin as a standard (Bradford, 1976).

Western Blot analysis

Equal amounts of proteins (20 µg) from RA treated samples and their controls in Laemmli sample buffer (1 M Tris-HCl, pH 6.8; 80% glycerol; 20% SDS; 2-mercaptoethanol; 0.1% bromphenol blue) were denatured at 100° C for 5 min., and allowed to cool to room temperature. The protein were then separated by SDS-polyacrylamide gel electrophoreses after adding Laemmli sample buffer . Proteins were

then transferred to nitrocellulose membranes by semi-dry electrotransblotting. Non-specific binding sites were blocked with 3% dried milk in PBS-Tween20 solution for an hour at room temperature. The membranes were incubated for two hours in PBS-Tween solution containing anti-human CEA antibody (Zymed Laboratories Inc., San Fransisco, CA). After two 15 min. washes, the membranes were incubated with a secondary antibody (goat anti-rabbit IgG) conjugated to alkaline phosphatase for two hours. Membranes were washed twice and developed using the BCIP/NBT (5-bromo-4-chloro-indolyl-phosphate/nitrobluetetrazolium, Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) substrate system that deposits a dark permanent color on phosphate bearing sites of the membrane. The amount of the antigen (CEA) was quantitated by densitometry.

ODC enzyme assay

Cells that are incubated with RA for various times were harvested and pellets were obtained as described. Pellets were homogenized in 50 mM sodium phosphate pH 7.2 buffer containing 5 mM dithiothreitol (DTT), 0.1 mM EDTA, 40 mM pyridoxal phosphate using a Dounce homogenizer. Lysates were centrifuged at 4°C for 10 min. Supernatants were removed as the protein sample. Protein determinations were calculated as described. The ODC activity was determined by a modification of the method of Djurhuus (1981) which is based upon the selective binding of putrescine to a strong cation exchange paper (Whatman P81), in the presence of 0.1 M NH_3. The assay aliquots composed of 300 ug of cell extract, 50 mM sodium phosphate buffer (pH 7.2), 5 mM DTT 0.2 mM pyridoxal phosphate, 0.2 mM L-ornithine and 34.6 nM L-[2,3-^{3}H] ornithine. The reactions were incubated at 37°C for 0-120 min. and at least three time points were taken (0.5, 1, 2 hours). Reaction were stopped by transferring samples to ice and applied to dry Whatman P81 cation exchange paper strips. Paper strips then were washed twice with 0.1 M NH_3 for the elution of unreacted ornithine. After elution, the paper strips were transferred to counting vials, dried, and counted. A control sample with the complete assay mixture but

without enzyme was run and not incubated at 37°C, but were applied to P81 paper and eluted together with the other samples. ODC specific activities (nmol putrescine/mg total protein) were calculated as a function of incubation time.

Gel retardation assay

The mobility shift (gel retardation electrophoresis) assay is based on the observation that DNA-protein complexes migrate through non denaturing polyacrylamide gels more slowly than unbound DNA fragments or oligonucleotides. The gel retardation assays were used for detection of AP-1 and Sp1 binding proteins in total cellular extracts of retinoid-treated HT-29 cells and their controls. The protocol uses double-stranded oligonucleotides whose sequence corresponds to the binding sequence of the regulatory region of genes capable of binding the protein transcription factors AP-1 or Sp1. Double-stranded AP-1 and Sp1 specific oligonucleotides (Gibco BRL, Grand Island, NY) were labeled by transferring the gamma phosphate of [γ-^{32}P]ATP to the 5'-OHs of the oligonucleotides by the action of T4 polynucleotide kinase. The labeled oligonucleotides were separated from unincorporated labeled substrate by mini-column chromatography (Stratagene, La Jolla, CA). Total cellular extracts were prepared as previously described. To allow binding, 10-20 μg of sample protein from various treatment conditions was incubated with the labeled oligonucleotide for 20 min. at room temperature in incubation buffer. The protein and labeled oligonucleotide samples were resolved on a homogeneous 20% non-denaturing gel using the Pharmacia Fast System (Uppsala, Sweden). The gels were wrapped in plastic and exposed to X-ray film at -80° C for 3 hours. HeLa nuclear extracts were used as positive controls.

PKC enzyme assay

Cells that were treated with RA for various incubation times were detached from culture flasks as described in previous sections and washed twice with PBS. Cytosolic

fractions of the cells were prepared by homogenization using a Dounce homogenizer in an isolation buffer containing 20 mM Tris-HCl, pH 7.5; 1 mM EDTA; 1 mM EGTA; 50 mM 2-mercaptoethanol; 1 mM PMSF; and 10 μg/ml leupeptin. The homogenates were sedimented at 100,000 x g for 90 min. at 4°C. Supernatant were removed as the cytosolic fraction. The pellets were resuspended in the above metioned isolation buffer containing of 5 mM CHAPS (3-[(-cholamidopropyl)-dimethylammonio]-1-propane-sulfonade, Sigma Chemical Co., St. Louis, MO) and incubated at 4°C for 2 hours. After centrifugation at 100,000 x g for 1 hour the supernatants were collected as the particulate fraction (solubilized membrane fraction). Protein concentrations were determined as described in the above sections. PKC activity was assayed by the method of Hass et al (1991), by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into calf thymus Histone-1 (Sigma Chemical Co., St. Louis, MO) in the presence or absence of lipids, 0.025 mg/ ml phosphatidylserine and 0.005 μg/μl 1,2-dioleoylglycerol (Sigma Chemical Co., St. Louis, MO). The reaction mixture contained 0.05 mg/ml sample protein; 20 mM Tris pH 7.5; 1 mM $CaCl_2$; 20 mM $MgCl_2$; 20 mM ATP; 100 mM [γ-^{32}P]ATP; 0.2 μg/μl histone (H-1). Assay reactions were carried out in multiwell plates in triplicates at 37°C for 5 min. The reactions were stopped by addition of 10% w/v chilled trichloracetic acid containing 1 mM ATP and 15 mM K_2HPO_4. The acid-precipitable materials were collected on nitrocellulose membranes and washed three times with 5% w/v chilled trichloracetic acid containing 15 mM K_2HPO_4. The membranes were then placed in scintillation vials with 2 ml of scintillation fluid and the radioactivity was measured in a scintillation counter. PKC activity was expressed as the difference between the values measured in the presence or absence of lipids in units of pmol of phosphate transferred to histone per min. per mg of sample protein.

Statistical analysis

Parametric tests were used for the statistical treatment of the data. Analysis of variance (ANOVA) was performed on both the individual experiments performed in each of MTT, ODC, and PKC assays, as well as on each experimental group as a whole. Least square difference (LSD) estimates were calculated where comparison of individual groups were needed. Results were expressed at significance levels $p < 0.05$ or lower using Student's t test and paired t test in concert with a priori post test, LSD.

CHAPTER 4

RESULTS

Retinoids inhibit proliferation of HT-29 cells

The inhibition of proliferation of HT-29 cells by retinoids was investigated using retinol, all-*trans*-RA, and 13-*cis*-RA with molar concentrations ranging from 10^{-10} to 10^{-5} during a 7-day-treatment period. The MTT assay was used to determine the growth rates of HT-29 cultures. Dose and time-dependent-effects of these retinoids on proliferation of HT-29 cells are shown in Figure 3. The data presented is the average of three separate experiments performed in triplicates. After day one, there were no difference in optical density of cell suspensions between untreated and treated cultures. Beginning from the third day of treatment, all three compounds exhibited inhibitory effects which were statistically significant ($p<0.05$) at concentrations of 10^{-6} M and lower with retinol, 10^{-5} M with all-*trans*-RA and 13-*cis*-RA. There were no significant differences in the inhibitory effects on the third day between these retinoids with the exception of all-*trans*-RA and 13-*cis*-RA which showed more inhibition at 10^{-5} M and 10^{-6} M then retinol. The lowest concentrations on the fifth day when statistically significant inhibitions were achieved were 10^{-7} M ($p<0.05$) for retinol and all-*trans*-RA ($p<0.05$), and 10^{-9} M ($p<0.01$) for 13-*cis*-RA. After seven days of treatment all three retinoids showed a significant ($p<0.05$) decrease in the proliferation of HT-29 cells with doses as low as 10^{-10} M. The range of inhibition achieved between the lowest and the highest concentrations at days 3, 5, and 7 were: 13 to 34%, 10 to 45%, 12 to 56% for retinol; 9 to 50%, 3 to 53%, 14 to 61% for all-*trans*-RA; 15 to 70%, 11 to 76%, 17 to 82% for 13-*cis*-RA respectively. Table 2 shows the retinoid-induced percent-inhibition of proliferation in HT-29 cells observed after 7 days of treatment.

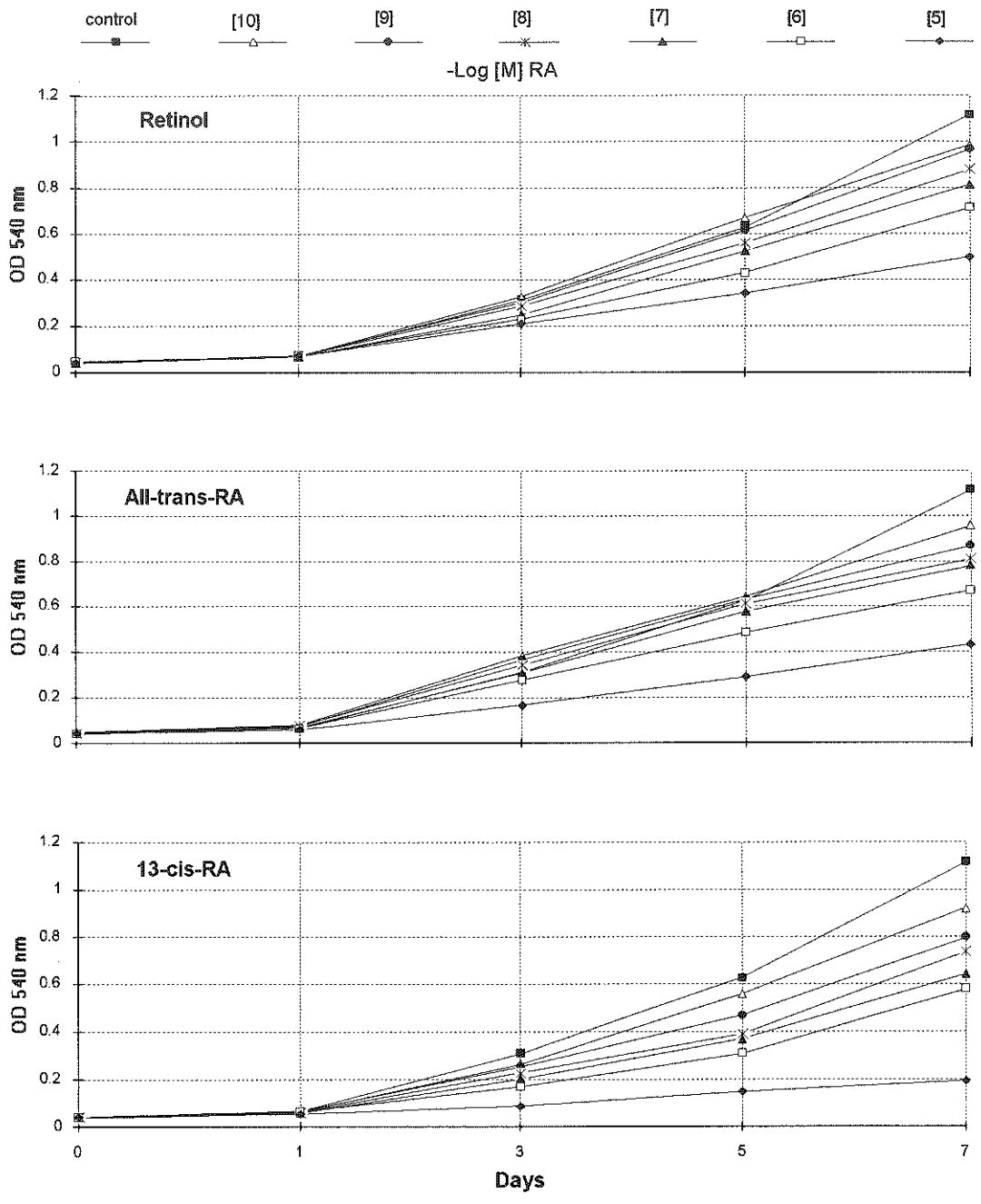

Figure 3. Dose and time dependent effects of retinol, all-*trans*-RA, and 13-*cis* RA on the proliferation of HT-29 cells. (Cells were incubated with retinoids for 7 days. Half of the medium was replaced with retinoic acid-containing medium everyday. Doses of RAs are given as -Log [M]. Cell proliferation was assessed as absorption readings of cell suspensions at 540 nm after incubating cultures with MTT for 4 hours.)

In the growth experiments 13-*cis*-RA was found to be the most effective inhibitory agent of the three retinoids tested. Although retinol and all-*trans*-RA significantly decrease proliferation within days, their effective concentrations were higher than that of 13-*cis*-RA. Based on these observations and evaluations 13-*cis*-RA was selected to be used as the principal retinoid to study the effects of retinoids on the growth and differentiation of HT-29 colon carcinoma cells specifically to quantitatively and qualitatively measure the regulation of expression of known colon carcinoma associated oncogenes, cell surface markers, enzymes and transcription factors that are involved in the regulation of these genes.

TABLE 2. RETINOIC ACID-INDUCED INHIBITION[1] OF PROLIFERATION IN HT-29 CELLS

	Molar Concentrations					
Retinoid	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
Retinol	5.3±0.57	7.4±0.36	16.0±0.51	21.7±0.58	31.9±0.81	52.3±1.16
All-*trans*-RA	17.7±0.78	25.3±1.05	30.2±2.23	33.2±0.81	42.2±0.81	62.7±2.02
13-*cis*-RA	17.3±1.29	28.7±2.34	34.1±1.71	42.4±2.97	53.2±2.54	82.4±5.02

[1] Percent inhibitions over control values within each concentration were computed as the difference between the normalized control and treatment groups ± SE. Calculations were based on the MTT assay data which represent the average of three experiments with triplicates after seven days of treatment.

Effect of retinoic acid on mRNA expression of ras oncogenes in HT-29 cells

Northern blot analysis of total cellular RNA was performed to determine the expression of the *ras* family genes which are known to be expressed by most of the human colon cancer cells. (Bos et al., 1987; Vogelstein et al., 1988). HT-29 cells were cultured in the presence or absence of 10^{-6} M RA for five days. Figure 4 shows the steady-state levels of expression of the Ha-*ras*, Ki-*ras*, and N-*ras* RNAs. There were no significant differences in the level of RNA expression of the three *ras* oncogenes between control cultures and RA treated cultures. Northern blot analysis were repeated in each case to confirm the negative results. Densitometric analysis of the scanned autoradiographs from separate experiments showed minute differences in the relative areas with respect to control bands. The levels of expression were statistically insignificant for all three *ras* oncogenes that were studied.

Effect of retinoic acid on carcinoembryonic antigen synthesis in HT-29 cells

There are very few biochemical markers that correlate with the state of differentiation in colon tumor cells. One such marker is the production of CEA. Therefore, the effect of RA on the production of CEA in HT-29 cells was tested. CEA antigen production was initially positive. HT-29 cells were treated with 10^{-6} M 13-*cis*-RA for 5 days. Figure 5 shows the Western blot analysis of CEA antigen synthesis. Treatment of the cells with RA did not induce CEA production at any time point tested. All six lanes, including the untreated (control) condition showed no bands in the Western blot of total cell lysates to anti-CEA at 180Kd, which is the molecular weight of this molecule. Equally dense bands observed below 180Kd could indicate the non-specific reaction between anti-CEA and other protein residues.

Figure 4: Northern blot analysis of Ha-*ras*, Ki-*ras*, and N-*ras* oncogenes in RA-treated HT-29 cells. Total cellular RNA from HT-29 cells (cultured for 5 days in the presence or absence of 10^{-5} M 13-*cis*-RA) were isolated using guanidinium thiocyanate cesium chloride ultracentrifugation method. Samples were separated through a 1.4% denaturing agarose gel electrophoreses. RNA was transferred to nylon membranes by capillary blotting. All three *ras* cDNA probes were end labeled with $\gamma^{32}P$ in the presence of T4 polynucleotide kinase. Hybridizations were performed at 42°C for 24hours in hybridization solution (0.1 ml/cm^2 of filter area containing 10% SDS, 1MNaCl, 50% deinonized formamide, 10% dextransulfate, and 100 mg/ml denaturedsalmon sperm DNA)containing 10^6 dpm/ml of denatured cDNA probes. Migration of 18S and 28S RNAs are indicated as internal molecular size standards. Sizes of transcripts are indicated in kilobases. Densitometric quantitation of the Northern blots were performed and relative expression of *ras* mRNAs (Y axis) are plotted versus time course of RA treatment (X axis).

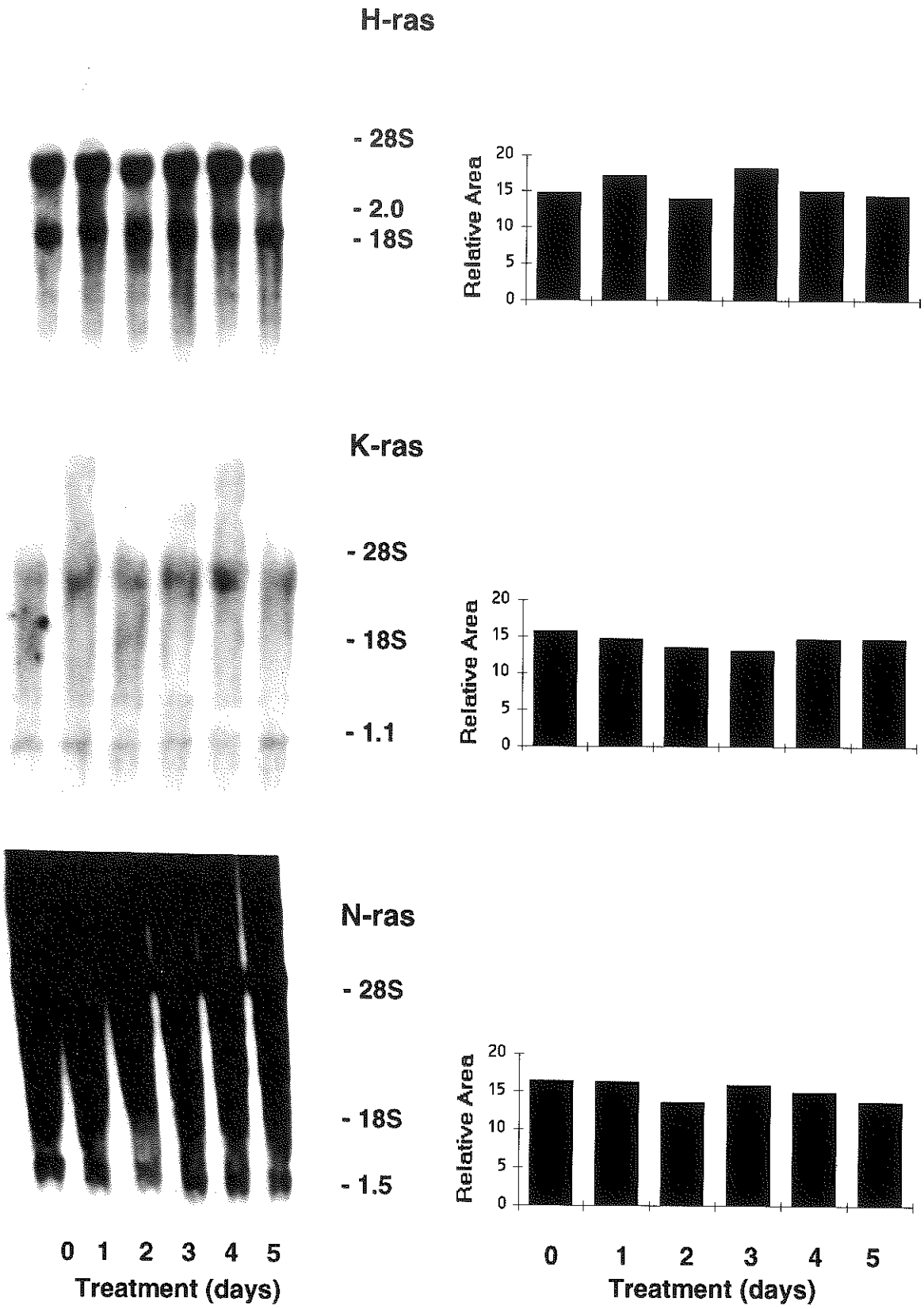

Figure 4. Northern blot analysis of *ras* oncogenes in RA-treated HT-29 cells.

20 ug protein from 13-cis-RA treated HT-29 total cellular extracts was seperated in SDS-PAGE electrophoresis, transfered to nitrocellulase membranes, and incubated with anti-CEA antibody. Days of treatment and MW markers are as indicated in kilodaltons (Kd)

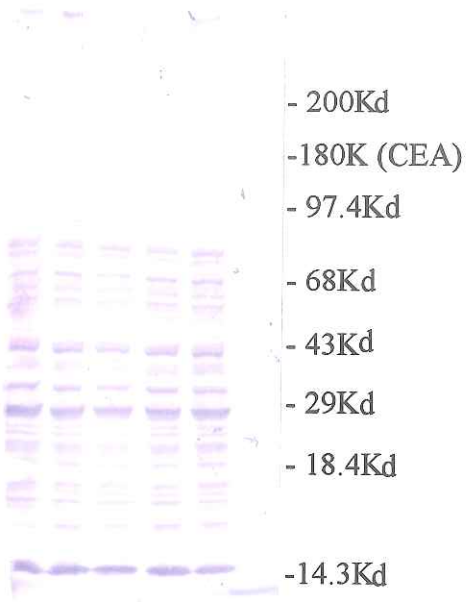

Figure 5. Western blot analysis of CEA synthesis in RA-treated HT-29 cells

MHC-class I expression

The effect of RA treatment on the antigenicity of HT-29 colon cancer cells was investigated by determining the extend of HLA-class I mRNA expression. Cells were incubated with 10^{-5} M 13-*cis*-RA for 7 days in parallel with control cultures which received ethanol alone. The experiment was repeated three times from separate cultures. The results of Northern blot hybridizations showed significant increase in the HLA-class I B7 mRNA expression. The patterns of expression were consistent in all three results. A representative autoradiograph is presented in Figure 6A. Densitometric quantitation of the blot (Figure 6B) showed a 2 fold increase in expression over control values after 3 days and more then 3 folds after 5 days of incubation with RA. There was a 20% decrease in the level of expression on the 7th day. This decrease may be due to a transient expression of the *class I* mRNA since treatment of the cells with RA terminated at day 5.

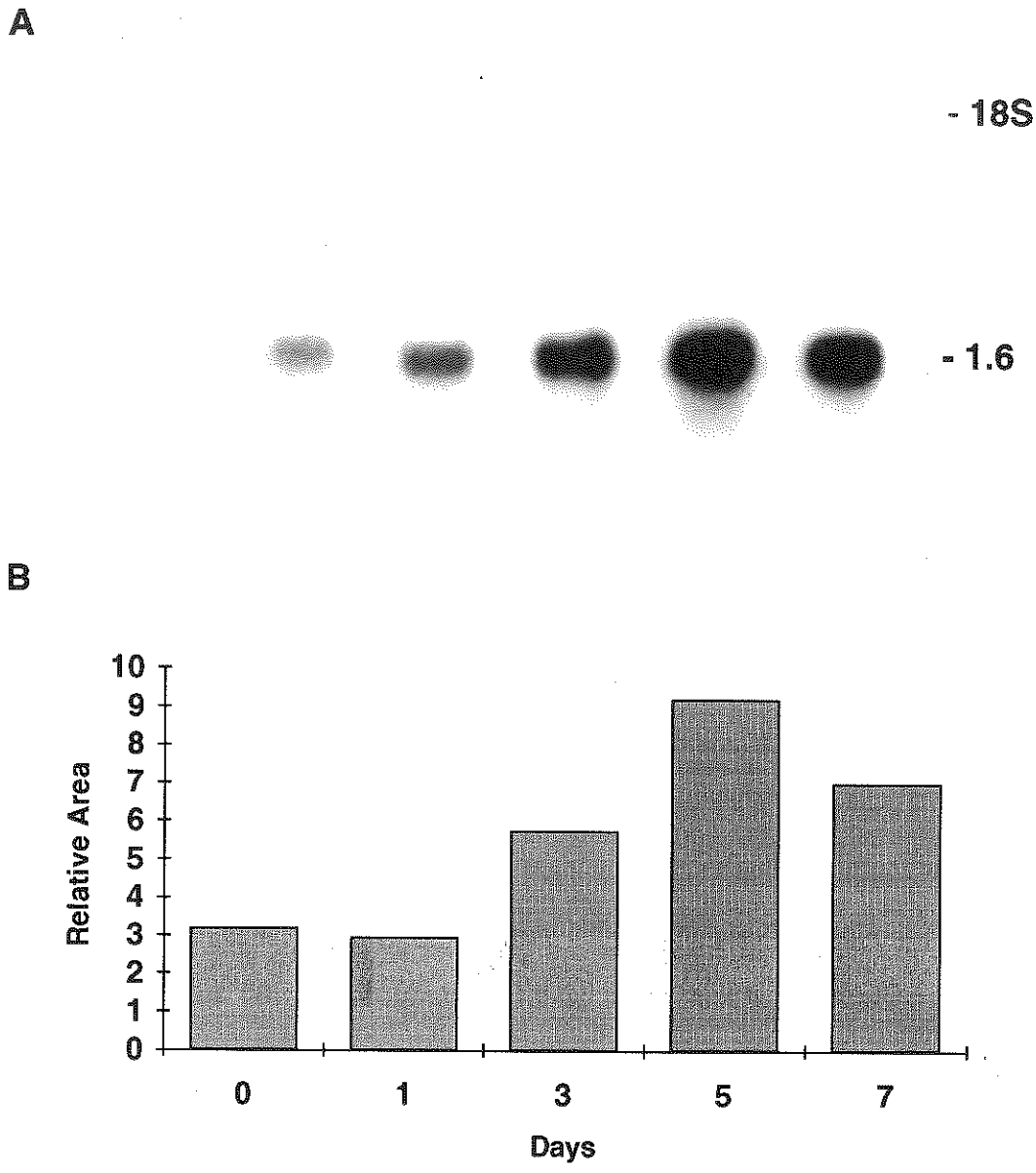

Figure 6. Northern blot analysis of HLA B7 mRNA expression from control and RA treated HT-29 cells (panel A). Cells were incubated with 10^{-5} M 13-*cis*-RA for the indicated days (0, 1, 3, 5, 7) and electrophoretically separated total RNA was hybridized with radiolabeled probe for HLA B7 mRNA. Migration of 18S RNA is indicated as internal molecular size standard. Densitometric quantitation of the Northern blot (panel A) is shown in (panel B). The relative expression of HLA-B7 mRNA (Y axis) is plotted versus time of RA treatment (X axis).

Regulation of ornithine decarboxylase synthesis

Several previous studies have reported elevated levels of ODC activity in neoplastic versus normal colonic mucosa (Hietala et al., 1990; Guillem et al., 1990; Elitsur et al., 1992). Its role as a key enzyme in the pathway of polyamine biosynthesis (Berdinskikh et al., 1991; Elitsur et al., 1992) and on the growth-inhibitory consequences of polyamine depletion (Kingsnorth et al., 1983) suggested that ODC down-regulation may be the mechanism by which RA inhibits proliferation of HT-29 cells.

HT-29 cells were cultured in the presence or absence of 10^{-5} M 13-*cis* RA for 5 days and the ODC activity was determined by a modification of the method of Djurhuus as described in Materials and Methods (Figure 7). One day posttreatment ODC activity was 45% higher than that of the controls. This induction is due to serum effect. Since ODC is known to be induced by the growth factors in serum. Day 2 enzyme activity was equivalent to the control value. After the 3rd day of the ODC activities were significantly lower than the control value even though fresh serum containing medium was added to the culture every 24 hours. The decreases were 23%, 47% and 77% of the control value on the 3rd, 4th and 5th days respectively. This assay was repeated three times and the differences between RA-treated and controls were statistically significant, 3.3 fold and 8.3 fold compared with controls and day 1 RA-treated samples.

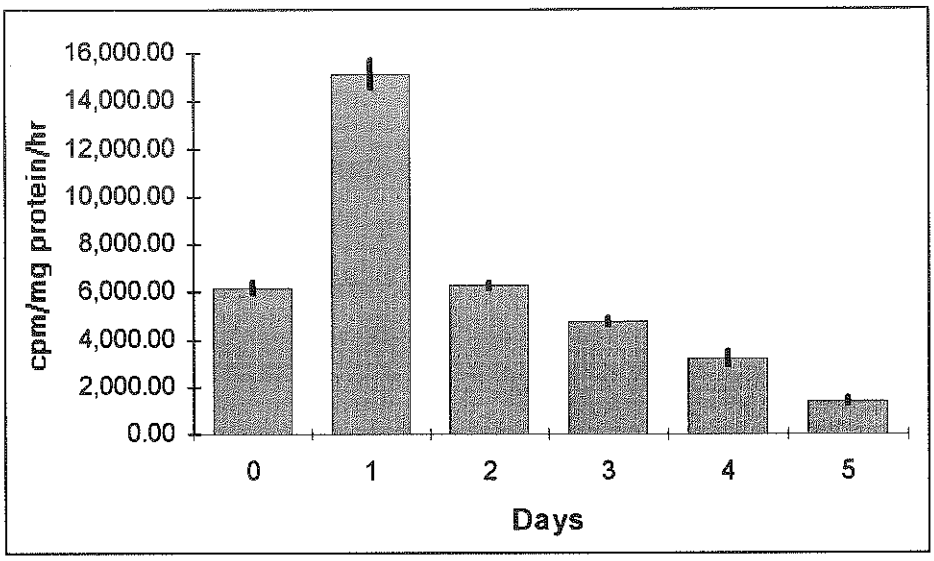

Figure 7. ODC activity in RA treated HT-29 cells. Total cellular extracts obtained from HT-29 cells treated with 10^{-5} M 13-*cis*-RA for indicated days (0, 1, 2, 3, 4, 5) were assayed for the enzyme activity as described in Materials and Methods. ODC specific activities (nmol putrescine/mg total protein) were calculated as a function of incubation time. Results are presented as arithmetic means from three time points of 0.5, 1, 2 hours reaction periods with standard error bars.

Total RNA was isolated from 13-*cis*-RA treated HT-29 cells in parallel to enzyme assay and probed with ODC cDNA as described in Material and Methods. Figure 8 shows the autoradiograph and densitometric scan of the Northern-blot. Steady state levels of ODC mRNA were elevated on the first day to 2.2-folds and on the second day to 1.4-folds of the control. On the third day mRNA expression was approximately equal to the control levels. However, 79% decrease was detected on the fifth day of the treatment.

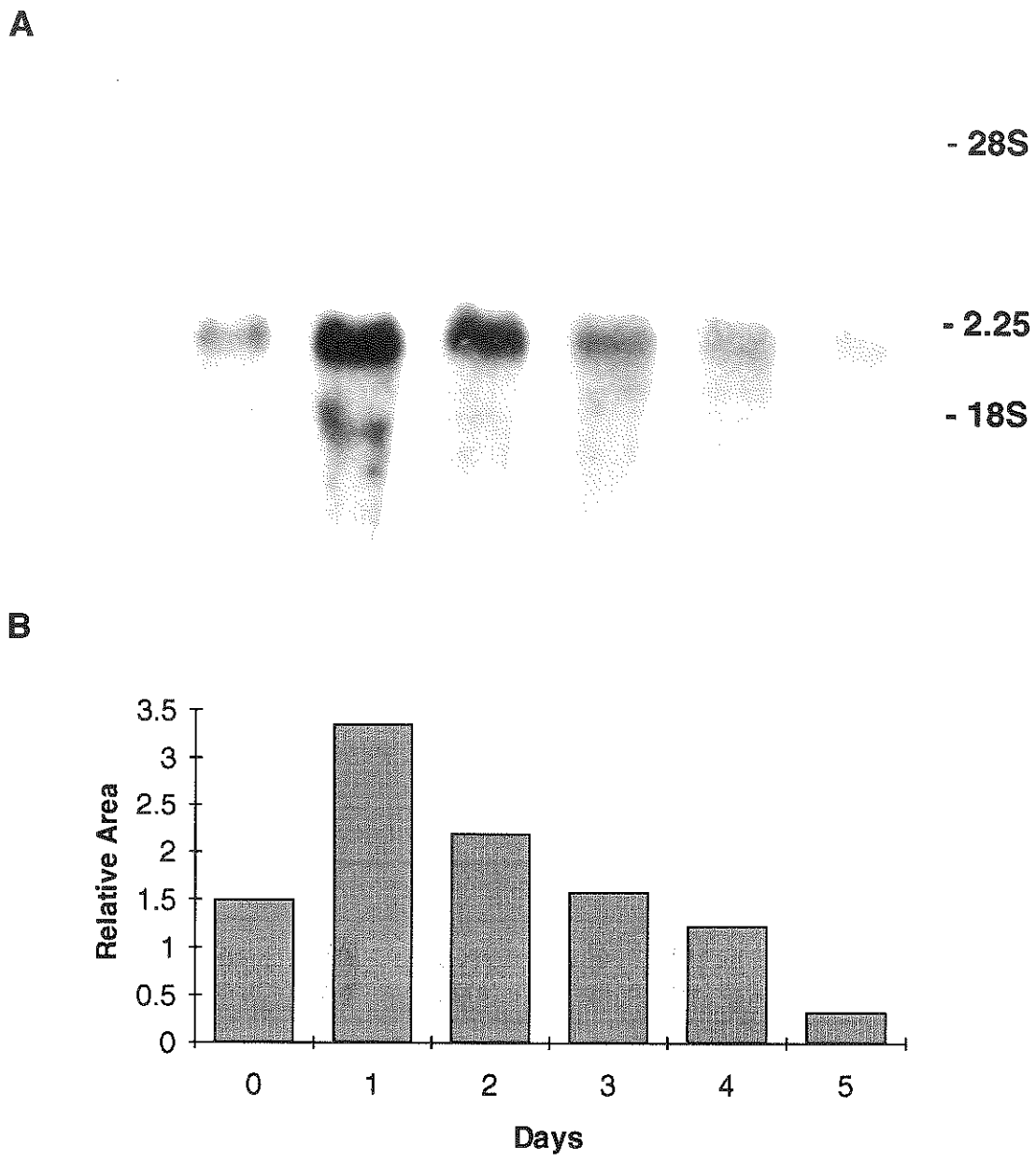

Figure 8. ODC mRNA expression in 13-*cis*-RA treated HT-29 cells (panel A). Cells were incubated with 10^{-6} M 13-*cis*-RA for the indicated days (0, 1, 2, 3, 4, 5) and electrophoretically separated total RNA was hybridized with radiolabeled probe for ODC mRNA. Migration of 18S and 28S RNAs were indicated as internal molecular size standards. Densitometric analysis of the ODC Northern Blot shown in panel A appears in panel B. The relative expression of ODC mRNA (Y axis) is plotted versus time of RA treatment (X axis).

Protein kinase C activity

Total PKC activity in HT-29 cells treated with 10^{-5} M 13-*cis*-RA for 0-96 hours increased by 35% at 24 hours, 76% at 48 hours, and more then 100% after 72 hours posttreatment. (Figure 9). Total PKC activity was the sum of the PKC activities measured from cytosolic and particulate fractions. Two-way analysis of variance (ANOVA) test was used to test three hypotheses (H1, H2, H3) that were: (H1) no difference in the enzyme activity among days; (H2) no difference in the enzyme activity among fractions; (H3) no interaction between time and source of fraction. All three hypothesis were rejected. Therefore, there is a time dependent difference in the PKC activity between the sources of the fractions.

TABLE 3. ANOVA[1] ON PKC ACTIVITY[2] IN HT-29 CELLS TREATED BY RA

Source of variation	SS	df	MS	Fs
Among fractions[3]	993344.4	1	993344.4	63.418*
Among days[3]	2708553	5	541710.5	34.584*
Interaction	1645237	5	329047.3	21.007*
Error	375921.3	24	15663.39	
Total	5723055	35		

[1]Two way with replication analysis of variance Model I on [2]Protein kinase C activity determined using either cytosolic or particulate fraction of each sample. [3]Values were obtained from one set of experiment in triplicates. *Significantly different at $F_{0.01\ (1,24)} = 7.82$ and at $F_{0.01\ (5,24)} = 3.90$. (SS=sum of squares; df= degrees of freedom; MS=mean square; Fs=F statistics)

To further investigate the statistical significance and directionality of the differences within the sources of the fractions the LSD (least square difference) test was used. The results of the LSD analysis showed that within cytosolic fractions there were no significant increases in the PKC activity between control and treatment values in the 5 day-treatment course except the 96 hour-sample. On the particulate side, all of the samples were found to have significantly higher PKC activities.

TABLE 4. PROTEIN KINASE C ACTIVITY IN HT-29 CELLS

	Activity (pmol/min/mg protein)*	
Condition	Cytosolic ± SE	Particulate ± SE
Control‡	4.01 ± 0.29	1.98 ± 0.53
Day 1†	3.96 ± 0.9	4.12 ± 0.43[a]
Day 2†	4.71 ± 0.6	5.81 ± 0.96[a]
Day 3†	4.77 ± 0.62	7.47 ± 0.87[b]
Day 4†	4.95 ± 0.61	15.29 ± 1.08[b]
Day 5†	6.34 ± 0.75,[a]	16.54 ± 1.44[c]

* PKC activity was determined using either cytosolic or particulate fraction of each sample. The values represent the average of pooled data from two separate experiments in triplicates. ‡Control values were obtained from 5-day cultures without RA treatment. The difference between 1-day and 5-day PKC activities in control cultures was statistically nonsignificant (data not shown). †LSD analysis performed as control vs. treatment pairs. [a,b,c]Significantly different with probabilities $p < 0.05$, $p < 0.01$, $p < 0.001$ respectively.

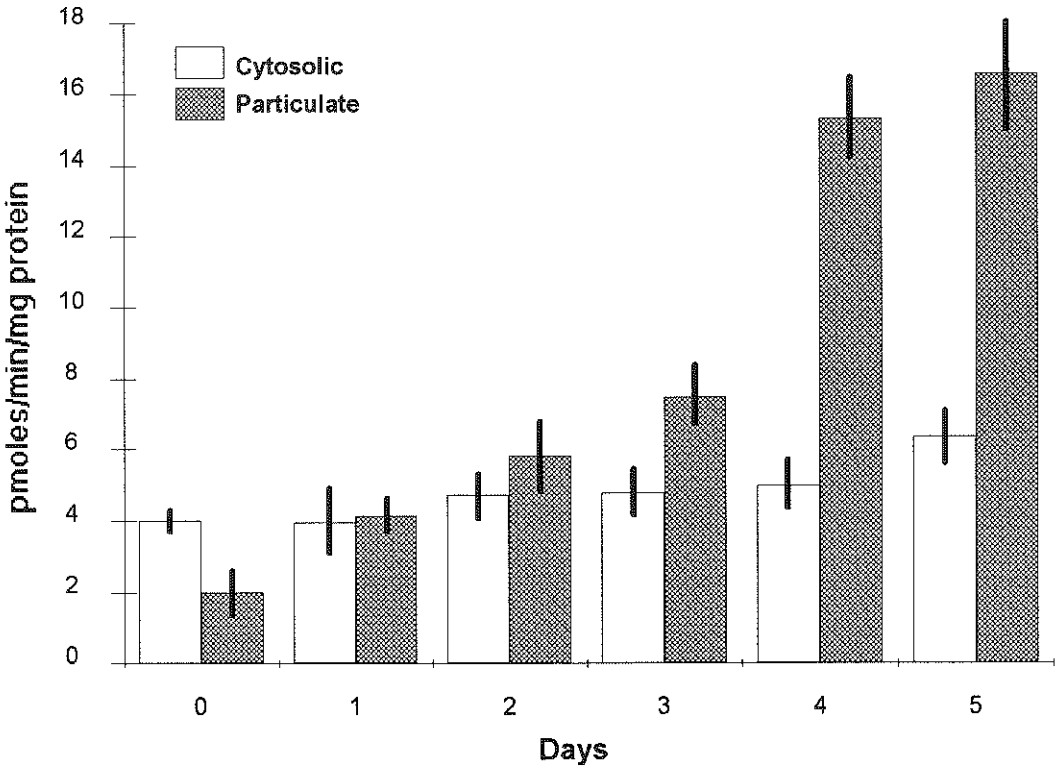

Figure 9. PKC activity in 13-*cis*-RA treated HT-29 cells. (Data obtained from Table 4)

TABLE 5. SUBCELLULAR DISTRIBUTION OF PROTEIN KINASE C ACTIVITY IN 13-CIS-RA TREATED HT-29 COLON CELLS

	Treatment condition (days)					
Fraction	control	1	2	3	4	5
% Cytosolic	66.9	49	44.8	39	24.5	27.7
% Particulate	33.1	51	55.2	61	75.5	72.3
% Translocation	0*	2	10.4	22	51	44.6

*Since control condition represents the initial status of the distribution of the enzyme activity from untreated cells the translocation is given as 0%.

Presented in Table 5 is the subcellular distribution of protein kinase C activity in HT-29 cells in the presence or absence of RA. The proportion of protein kinase C activity from the particulate fraction showed an increase beginning at 24 hours of incubation with RA. This observation indicated that the activation of PKC in HT-29 treated with RA involves translocation of PKC from the cytosol to the membrane rather than covalent modification. The translocation was at its maximum after 4 days of treatment as 51% the activity in the cytosolic fraction was translocated to particulate fraction. Furthermore, the total PKC content was increased in RA-treated cells indicating that RA also induced new protein synthesis particularly in the particulate fraction.

Retinoic acid induced regulation of transcription factors AP-1 and Sp1

Schule et al. (1991 a,b) recently observed that RA can act as a negative regulator of AP-1 responsive genes, and suggested that this could occur via the formation of a

nonproductive complex between RAR and AP-1 transcription factor. In order to investigate whether RA treated HT-29 cells have down-regulated transcriptional activity as a result of interference by the RAR AP-1 gel retardation assays were performed. HT-29 cells were cultured in the presence and absence of 13-*cis*-RA for seven days. HT-29 cell extracts that were obtained from control, 1, 3,5 and 7 days along with HeLa cell extracts (positive controls) were incubated with labeled oligonucleotide probe specific for AP-1. The formation of AP-1 - DNA complex was observed after the electrophoretic separation (Figure 10A). The first lane of the autoradiograph shows the complex formed with AP-1 binding site positive HeLa nuclear extracts and AP-1 oligonucleotide. The second lane also shows the complex which was formed with untreated HT-29 cell extract and AP-1 oligonucleotide. Beginning from the first day of treatment there were a gradual decrease in the formation of the complexes which can be seen on the remaining lanes to the right at days 1, 3, 5, and 7.

Sp1 transcription factor is known for its role in the control of differentiation-specific expression of genes like F9 teratocarcinoma cells under the influence of RA (Darrow et al., 1990). The transcriptional activity of Sp1 was observed in gel retardation assays in HT-29 colon cancer cells in the presence or absence of 13-*cis*-RA in the culture medium (Fig 10B) . In the first lane the complex between the oligonucleotide specific for Sp1 and Sp1 itself is shown in the HeLa nuclear extracts. Untreated HT-29 cell extracts on the other hand showed a very light band under similar conditions. The third and the fourth lanes which correspond to one day and three day treated conditions presented similar intensities. After 5 days of incubation with RA there were significant increase in the intensity of the bands which indicated the presence of Sp1 transcription factor (lane 5 and 6).

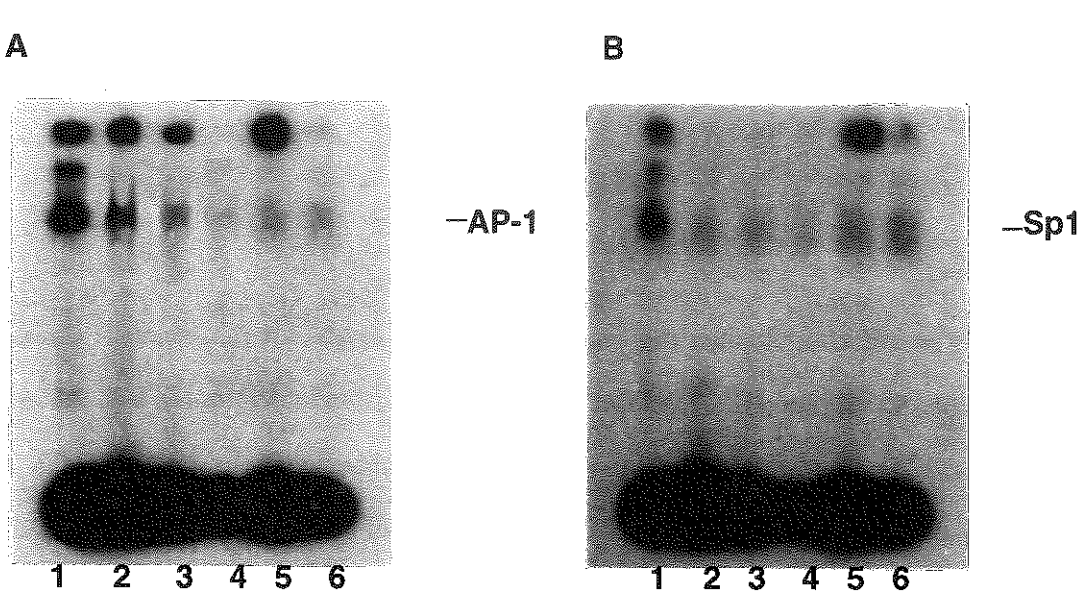

Figure 10. Gel retardation assays of AP-1 and Sp1 transcription factors. The labeled AP-1 (panel A) and Sp1 (panel B) specific oligonucleotide probes were incubated with 5 ug of total cell extract obtained from either HeLa cells (lane 1 in both panels) or untreated HT-29 cells (lane 2 in both panels) or 1, 3, 5, and 7-day 13-*cis*-RA treated HT-29 cells (lanes 3, 4, 5 and 6 in both panels) and electophoresed on 20% homogeneous native polyacrylamide gel and visualized by autoradiography. Indicated in both panels are the complexes formed with AP-1 and Sp1 and their response elements.

CHAPTER 5

DISCUSSION

The present study was undertaken to gain more insight into the as yet incompletely understood antitumor mechanism of action of retinoids in colon cancer cells. Retinoids are a class of compounds which have been reported to induce differentiation and/or suppression of proliferation in a range of transformed cells. The antitumorigenic effect of retinoids has been investigated in tumor models such as embryonal carcinoma (Miller et al., 1990), leukemia (Lippman et al., 1987), melanoma (Amos et al., 1989; Lotan, 1986), neuroblastoma (Sidell, 1982), breast cancer (Fontana, 1987; Halter et al., 1988), as well as epithelial malignancies (Lippman et al., 1989).

The results of the growth experiments in the presented work showed a profound antiproliferative effect of retinoic acid (RA) on HT-29 human colon cancer with inhibition rates of up to 82% using nontoxic doses of the compound. Although all the retinoids, retinol, all-*trans*-RA, and 13-*cis*-RA inhibited the growth of the cells in culture, 13-*cis*-RA was more effective than the other two forms, showing over 50% and 30% inhibition when compared to the native forms of the vitamin, retinol, and all-*trans*-RA respectively. The growth inhibitory effect of 13-*cis*-RA *in vitro* was achieved in a dose range of 10^{-10} - 10^{-5} M which is physiologically relevant concentrations of RA. Indeed, a concentration of 10^{-5} M of 13-*cis*-RA correlates with a normal human plasma level. In control cultures cells began to adhere to the bottom of the culture dish after 24 hours of incubation and formation of the colonies was observed. However, the colony forming efficiency of 13-*cis*-RA-treated cells was remarkably reduced. It is reasonable to assume that the reduced colony formation is an indication of RA-mediated inhibition of proliferation in HT-29 cells.

As a first step toward understanding the molecular basis of the growth inhibitory effect of 13-*cis*-RA in the colon cancer cells, the expression of proliferation and differentiation related oncogenes H-*ras*, K-*ras*, and N-*ras* were examined. These genes were chosen for analysis because of their documented involvement in the molecular sequence of events leading to colon cancer where their mutations in the cellular genome affect the expression or function of genes controlling cell growth and differentiation. Results of this study showed no significant change in the steady-state levels of the H-*ras*, K-*ras*, and N-*ras* mRNAs in HT-29 cells following 13-*cis*-RA treatment. In fact this observation is in accord with the reported studies of the *ras* family oncogenes in RA-mediated differentiation of teratocarcinoma cell line NT2/D1 (Miller et al., 1990). Thus it may be suggested that the growth inhibition effect of RA is not related to the inhibition of growth regulation of *ras* oncogenes in HT-29 cells.

One of the properties of human colon cancer cells is their ability to produce carcinoembryonic antigen (CEA). In general, the tissue and serum levels of CEA in colon cancer patients reflect the state of differentiation of the tumor, with higher CEA contents being characteristics of the well differentaited tumors, while poorly differentiated or anaplastic tumors have low or no CEA content. Sodium butyrate, a maturation inducing agent, was reported to enhance CEA production in some but not all colon cancer lines (Tsao et al., 1983). RA was shown to increase CEA release in a human gastric adenocarcinoma cell line (Kobayashi et al., 1990) but the effect of retinoic acid on CEA production in colon tumor cells has not been studied except one report in which it had no effect on the CEA content of HRT-18 cells (Tsao et al., 1983). Therefore, it was examined as to whether CEA production was affected by the putative differentiation agent RA. The results of the present study showed that retinoic acid did not enhance the production of CEA in HT-29 cells. Since the differentiation state of the tumor cell is related to the CEA content of the cell, perhaps the reason why retinoic acid fails to induce CEA production in HT-29 cells is that these cells are moderately differentiated. The

mechanism of the biological effect(s) of RA on the tumor cells with different differentiation stages is unclear and yet to be studied.

An important finding of the present study which is a well characterized marker of phenotypic normalcy in terminally differentiated colon cells is that RA induces the HLA class I antigen mRNA expression in HT-29 cells. Although this finding cannot be used to explain the antiproliferative activity of RA on colon tumor cells, it has to be evaluated in the broad terms of RA-action on tumor cells. Reduced expression of class I HLA has been suggested to be associated with increased malignancy of tumor cells (Garrido et al., 1986). The basic idea concerning the role of class I molecules in oncogenesis is that, since they are required in the presentation of neoantigens on tumor cells to the cytotoxic T-lymphocytes, their absence from the cell surface may lead to escape of these tumors from immunosurveillance. Although studies have been reported the effect of various antitumor substances on the expression and modulation of HLA in tumor cells *in vitro* and *in vivo* (Mohla et. al., 1990; Baghdiguianet al., 1992), there are limited number of studies in the literature on the effect of retinoids on HLA expression in tumor cells and neither of them focus on the colon tumor cells.

The data of this study also indicated that 13-*cis*-RA down regulates the expression of ornithine decarboxylase (ODC) mRNA expression and reduces enzyme activity in HT-29 cells. ODC is a key enzyme in cell proliferation through its involvement in the biosynthesis of polyamines which have been shown to modulate gene expression in COLO 320 human colon carcinoma cells mediated by a decrease in the transcription rates of c-myc and c-*fos* (Celano et. al., 1989). The effect of polyamines on the transcription of genes has been explained by several mechanisms including changes in structure and conformation as well as chromatin and nucleosomal changes in DNA through its ionic interactions to influence binding of transcription regulatory factors (Tofilon et al., 1982; Elgin, 1988). The observations from the present work lead to the suggestion that the 13-*cis*-RA- reduced ODC levels might mediate the effect of RA on the inhibition of

proliferation, and induction of HLA mRNA as an indication of differentiation which is the subsequent step in the normal cell life span.

The results presented in this report demonstrate that 13-*cis*-RA increased protein kinase C (PKC) enzyme activity in HT-29 cells. The enzyme activity in cytosolic fraction was not altered. In contrast the proportion of PKC activity associated with the particulate fraction was clearly higher in the RA treated cells than in the control cells. This finding implies that a greater proportion of the enzyme activity in the cells was in an activated state. Thus, PKC forms a complex with Ca and phospholipid and translocates from cytosolic to particulate fraction when exposed to RA. PKC has seven isoforms which three of them have been well characterized (Ohno et al., 1991). Studies showed that RA-induced PKC isoforms are expressed in a cell type and tissue specific manner (Tonini et al., 1991) and is involved in the transduction of many different extracellular signals, ranging from hormones to neurotransmitters, antigens, and growth factors. In several instances, PKC transduction of multiple signals appear to be contradictory. For example, overexpression of PKCβ1 in a rat fibroblast cell line led to an increase in growth as well as an increase in saturation density, and also resulted in the ability to form dense foci and to grow in soft agar (Housey et al., 1988). Also, overexpression of PKCγ in NIH 3T3 fibroblasts resulted in increased tumorigenicity (Persons et al., 1988). Conversely, overexpression of PKCβ1 in colon cancer cells led to a decrease in growth and tumorigenicity (Choi and Weinstein, 1991). In the HT-29 cells, PKC appears to act in a manner similar to latter case where an increase in PKC activity is associated with decreased growth and may have a role in mediating growth inhibitory effects of RA.

Possible mechanisms of retinoid action

Retinoids can alter the phenotype of different cells in numerous ways. administration of these compounds to cells has been reported to influence the synthesis of

almost every class of macromolecules; numerous enzyme activities have been found to be modulated; and membrane integrity and cell surface structure are modified. Such changes often accompany profound modulation of the growth and/or differentiation states of cells. It is reasonable to assume that some of the molecular changes might be responsible for, whereas others might be a consequence of, these alterations in cellular behavior; however, in most instances one cannot separate cause from effect.

In efforts to elucidate the mechanism or mechanisms by which retinoids elicit their effects, the initial step can be the assumption that there is a single fundamental pathway by which retinoids ultimately promote or induce a series of macromolecular changes that influence the state of cell behavior. The first issue that may be considered is whether retinoids act in the same way to influence growth and differentiation. In most instances the proliferative ability of cells is dramatically reduced when cells reach their final differentiated phenotype. It is not surprising, therefore, that as retinoids induce cell differentiation there is usually a concomitant decrease in growth rate. But it is also possible that agents can influence differentiation indirectly through a primary effect upon growth. For example, embryonal carcinoma cells cultured in the presence of an autocrine growth factor become refractory to differentiation (Jakobovits and Martin, 1984). Conversely, inhibitors of DNA replication can promote differentiation of embryonal carcinoma cells (Nishimune et al., 1984). In some serum-free culture free media, embryonal carcinoma cells tend to undergo spontaneous differentiation at high frequency (Rizzino, 1983); this might suggest that even a subtle retardation of the growth rate of these cells without a direct block in RNA and protein synthesis could trigger the cells to move into a differentiative mode.

One could argue that retinoids suppress growth of cells by directing them into a more differentiated state or that retinoids indirectly promote differentiation by suppressing proliferation. In either case, a single mode of action could lead to modulation of both phenomena. Eventually, this issue might not be resolved until we have a clearer

general understanding about the relationship between growth and differentiation. If the pleiotropic effects of retinoids are elicited via a single triggering mechanism, what is the likely mode and site of action? Based upon contemporary concepts of cell regulation, the two most likely sites of action are the cell membrane and the nucleus. Action of retinoids at the level of the plasma membrane is an attractive view because it could explain resultant changes in cell shape and interaction and, via second-messenger activity, alterations in gene expression. It was proposed that retinoids regulate gene expression indirectly through interaction with cyclic-AMP-dependent and/or calcium-dependent protein kinases (Sporn and Roberts, 1983). There are several studies documented which suggest that retinoid treatment enhances protein kinase activities in embryonal carcinoma, HL-60 promyelocytic leukemia and melanoma cells, and which demonstrate that cyclic AMP potentates retinoid-induced differentiation of embryonal carcinoma and HL-60 cells (Hashimoto et al., 1990; Tanaka et al., 1992).

The discovery of the nuclear retinoic acid receptors (RARs) have been a major step towards elucidating the mechanism of retinoids' actions. Although it seems that RARs are the ultimate mediators of the action of retinoids on gene expression, direct proof for that contention is available in only a few cases. One example of supportive evidence is the finding that the biologic activity of several retinoids correlates well with their affinity for the nuclear receptors (Darmon et al., 1988). More direct evidence for RARs' function has come from studies with leukemia cells. RARs were identified in HL-60 promyelocytic leukemia cells, which are induced to undergo myeloid differentiation by RA (Hashimato et al., 1989). An RA-resistant mutant subclone of HL-60 cells was found to contain a defective RAR-α. That this defect was the cause for resistance to RA was indicated by the demonstration that transduction of a single copy of RAR-α *via* a retroviral vector into the mutant cells restored responsiveness to RA (Collins et al., 1990).

Although the above results strongly support a direct role for RARs in mediating RA actions, the presence of RAR-α and RAR-γ and the induction of RAR-β are not

sufficient to render a cell responsive to the growth inhibitory or differentiation-inducing effects of retinoids. For example, RAR-α is expressed by most human leukemias regardless of their responsiveness to RA (Gallagher et. al., 1990). Similar results were found for RAR's constitutive expression and inducibility in murine PCC4aza1R embryonal carcinoma cells and two RA-resistant mutants derived from them (Nervi et al., 1990). It appears that RARs may be necessary but not sufficient for mediating the effects of retinoids on gene expression. Other proteins such as nuclear transcription factors (e.g. c-*jun)* may be required for the formation of a transcriptionally competent complex among RAR, polymerase, and DNA. Such factors may be defective in some RA-resistant cells that contain normal RARs. Alternatively, the RAREs of some target genes for RA-regulated genes may be defective in resistant mutants. Finally, resistance to retinoids may be the result of defects in "postreceptor" events (e.g. rapid metabolism of the retinoid before it has a chance to bind to nuclear RARs) or in "'postreceptor" events (e.g. the regulation of posttranscriptional events).

An important discovery with respect to retinoic acid was that *Jun-Fos* (AP-1) and the RAR-α recognize a common response element in the human osteocalcin gene the actions of RAR-α and *Jun-Fos* proved to be antagonistic at this AP-1 site (Schule et al., 1991). Recent evidence suggests that there may be additional mechanisms of gene control by retinoid receptors, apart from direct DNA binding. Thus, it has been shown in several laboratories (Schule and Evans, 1991a) that there is reciprocal repression of transcriptional activation by the glucocorticoid receptor and members of the *Jun-Fos* (AP-1) complex, mediated by protein-protein interactions rather than by direct DNA binding. Based on these results, it may be suggested that expression of certain genes that are, regulated by retinoic acid might also be controlled by protein-protein interactions between RAR-α. and the *Jun-Fos* (AP-1) complex. Taken together, these new studies demonstrate that there will be multiple levels of cross-coupling of transcriptional control by members of the steroid receptor and leucine-zipper classes of transcriptional regulators (Schule and

Evans, 1991a). These mechanisms directly couple these two major signal transduction systems and provide a basis for integrated control of gene expression by growth factors, steroids and retinoids, and oncogenes.

Because retinoids exert this hormone-like control of cell differentiation and cell proliferation, and particularly because retinoids also reverse premalignant epithelial lesions and suppress malignant transformation, it is logical to investigate whether they might also be useful agents for chemoprevention of carcinogenesis in various organs *in vivo*. This basic approach thus involves an enhancement of the intrinsic physiological mechanisms that protect the organism against the development of mutant clones of new cells that could potentially invade and destroy it (Cairns, 1975; Lippman et al., 1987). Without such intrinsic defense mechanisms, the incidence of invasive cancer would be much higher, since humans are constantly exposed to both endogenous and exogenous mutagenic agents. Since the process of carcinogenesis is fundamentally characterized by loss or arrest of cellular differentiation and growth control, and since retinoids both suppress growth and induce or enhance cellular differentiation, chemoprevention of cancer with retinoids represents a physiological, rather than cytotoxic approach to arrest or reverse the process of carcinogenesis.

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