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EFFECT OF RETINOIDS AND PEPTIDE GROWTH FACTORS
ON ANCHORAGE INDEPENDENT GROWTH BY
HUMAN FIBROBLASTS

presented by

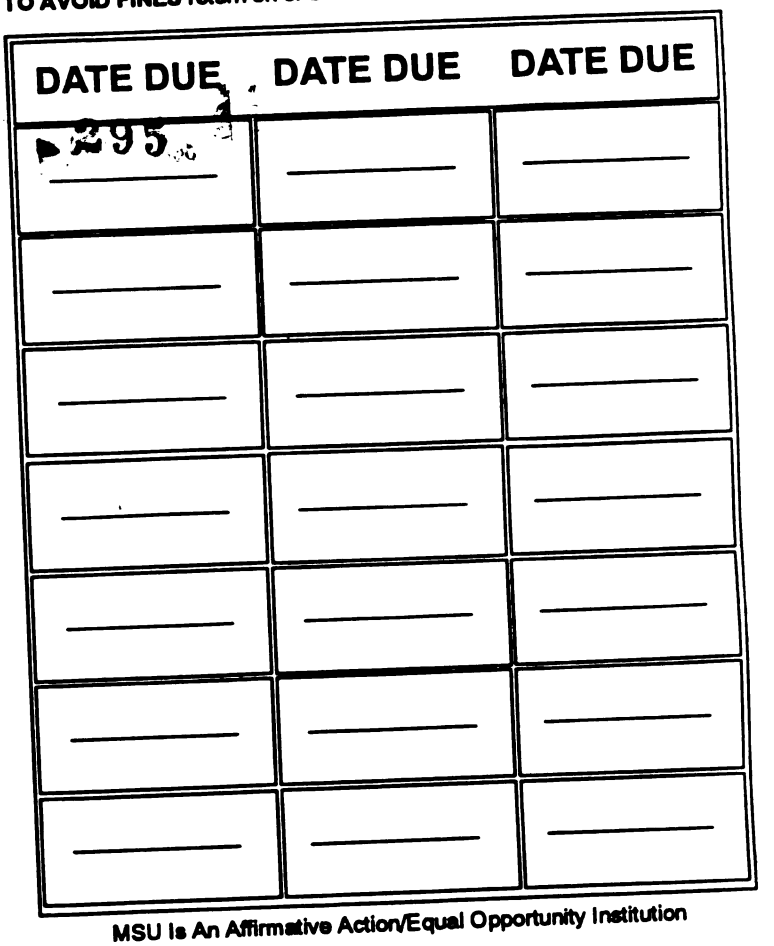
Helen J. Palmer

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science and
Human Nutrition

Major professor

Date Nov. 11 1988



EFFECT OF RETINOIDS AND PEPTIDE GROWTH FACTORS ON ANCHORAGE INDEPENDENT GROWTH BY HUMAN FIBROBLASTS

by

Helen J. Palmer

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Food Science
and Human Nutrition

1988

ABSTRACT

EFFECT OF RETINOIDS AND PEPTIDE GROWTH FACTORS ON ANCHORAGE INDEPENDENT GROWTH BY HUMAN FIBROBLASTS

By

Helen Palmer

Fibroblasts transformed in culture acquire phenotypes characteristic of tumor cells, such as anchorage independence, a phenotype highly correlated with the tumorigenicity of rodent fibroblasts. Normal human fibroblasts ordinarily do not exhibit this phenotype, but it can be induced by high levels of serum. Since serum contains peptide growth factors, one or more of these may cause anchorage independence. The experiments reported here were designed to determine which peptide growth factors would induce anchorage independent (AI) growth of human fibroblasts and to determine the effects of various retinoids on this growth. A serum-free medium was used to test the ability of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), or transforming growth factor-β (TGF-β) to induce AI growth in the absence of serum. None of the growth factors alone or in combination induced AI growth in this medium, although it supports monolayer growth of fibroblasts. Since no growth was induced in serum-free medium, medium supplemented with serum that had had its peptide growth factors inactivated by treatment with dithiothreitol and iodoacetamide was used.

In this medium, designated GFIS, AI growth was induced by PDGF or the basic form of fibroblast growth factor (bFGF), but not by EGF or TGF-β. EGF with TGF-β, at concentrations which were ineffective when tested alone, induced AI growth, but the frequency of colony formation was five times lower than the frequency induced by PDGF or 10% serum. The ability of non-cytotoxic concentrations of the retinoids to reduce the growth factor-induced AI growth was tested. All-trans retinol (0.5 uM) decreased the AI growth induced by PDGF or bFGF in GFIS. All-trans retinoic acid (1.0 nM) but <u>not</u> anhydroretinol, a biologically inactive retinoid (1.0 uM), also decreased the frequency of this growth induced by PDGF as well as that induced by 10% serum. All-trans retinol also decreased the frequency of AI growth of a human fibrosarcoma-derived cell line which grew in GFIS without added growth factors. The results showing that all-trans retinol decreases the frequency of AI growth induced by either PDGF or bFGF and the fact that these growth factors have different mechanisms for transmitting the signal for DNA synthesis in rodent fibroblasts, strongly suggest that all-trans retinol interferes with a common step late in the signal transduction pathway.

To

my family
including George, Fred, Dawn, Marian
and Crystal, Greg, Glen, Roxana, Jamie and J.J.

and

to the special people at URC

ACKNOWLEDGMENTS

I want to thank Drs. Justin McCormick and Veronica Maher for the knowledge and training that I received from them while working in their lab.

I also want to thank the rest of my committee: Dr. Dale Romsos, Dr. John Speck, Dr. Clifford Welsch and Dr. Wanda Chenoweth for each of their contributions at various times throughout my program.

In addition, I'd like to thank the many people and friends that have provided support and encouragement to me during my program.

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ABBREVIATIONS

ATP - adenosine triphosphate

bFGF - basic fibroblast growth factor

BSA - bovine serum albumin

DMBA - 7,12-dimethylbenz[a]anthracene

EGF - epidermal growth factor

FBS - fetal bovine serum

GDP - guanosine diphosphate

GFIS - growth factor-inactivated serum

GTP - guanosine triphosphate

HPRT - hypoxanthine-guanine phosphoribosyl transferase

LTR - long terminal repeat

MNU - N-methyl-N-nitrosourea

NRK - normal rat kidney

ODC - ornithine decarboxylase

PDGF - platelet-derived growth factor

RA - all-trans retinoic acid

Rol - all-trans retinol

SSV - Simian sarcoma virus

SV-40 - Simian virus 40

TGF-β - transforming growth factor-β

TPA - 12-O-tetradecanoylphorbol-13-acetate

INTRODUCTION

Cancer is the second leading cause of death in the United States. Although the most recent cancer statistics indicate that mortality caused by cancer has been on the decline since 1950, the overall incidence has increased (NCI, 1988). Exposure to environmental factors, such as man-made and natural chemicals, viral infections and diet, can affect the risk of cancer (Doll and Peto, 1981). The contribution of diet to the development of various forms of cancer has been estimated to be 10% - 70% by Doll and Peto (1981) and 40% - 60% by Wynder and Gori (1977). Some components of the diet can be protective against certain types of cancer; whereas, other components are associated with increased incidence of some cancers (reviewed by the Committee on Diet, Nutrition, and Cancer/ NRC, 1982). Among the macronutrients, consumption of a high level of fat, particularly saturated fat, is associated with increased incidence of breast and colon cancer (Hopkins and Carroll, 1979; Sylvester et al., 1986; Reddy et al., 1977), while some forms of fiber are protective against colon cancer (Wilson et al. 1977; Barbolt and Abraham, 1978). Among the micronutrients, vitamin C has been reported to be protective against stomach cancer (Kolonel et al., 1981); selenium has been shown to decrease the incidence of chemically induced experimental cancers (Ip and Sinha, 1981); vitamin E, an antioxidant, appears to protect against

cancer that develops from exposure to a carcinogen that requires oxidative metabolism to an active metabolite (Fiddler et al., 1978); and carotenoids and vitamin A may be important in protection against lung and other forms of cancer (reviewed in Chapter 1, section III).

Vitamin A belongs to a class of compounds referred to as retinoids (see Appendix A). The value of retinoids, particularly vitamin A, as inhibitors of cancer has been the subject of extensive research. In general, their ability to retard or prevent cancer development has been shown by epidemiologic studies, experimental studies using animal models, and clinical trials (Sporn and Newton, 1979; Bollag and Hartmann, 1983; Moon et al., 1983). The evidence will be discussed in Chapter 1, Section III.

In spite of the reported effectiveness of retinoids as inhibitors of cancer, topical treatment or dietary supplementation with retinoids can not be generally recommended as a preventive or curative measure. This is because many retinoids have toxic side effects (Kamm et al., 1984; Turton et al., 1985) and have sometimes been shown to enhance experimentally induced cancers (Polliack and Levij, 1969; Hennings et al., 1982; McCormick et al., 1987). In order to understand the possible usefulness of retinoids as therapeutic or preventive agents with respect to cancer, it is necessary to know more about their mechanism of action and about the mechanisms involved in the development of malignant tumors.

The development of cancer is considered to be the result of a multistep process. In vivo experiments on skin cancer in mice indicated a two stage model involving an irreversible first stage followed by a

second, latent stage resulting in the development of papillomas (Berenblum, 1940; Berenblum and Shubik. 1947). Progression, a third phase or stage, is the transition to malignancy and the ensuing period of tumor growth during which further changes in the character of the tumor may occur (Foulds, 1957).

The multistep nature of carcinogenesis in vivo has been extended to cell culture systems. Using oncogene transfection, Land et al. (1983) and Land et al. (1986) have shown that in rat embryo fibroblasts more than one permanent change in a cell is necessary for the cell to become malignantly transformed. The mechanisms that can result in these changes and the nature of the changes are not completely understood.

One of these changes for fibroblast cells results in aquisition of anchorage independence, the ability to grow in semisolid medium. In rodent fibroblasts, this phenotype has been shown to exhibit a high correlation with tumorgenicity (Freedman and Shin, 1974; Shin et al., 1975). Non-transformed human fibroblasts do not express such a phenotype unless assayed in medium containing high concentrations of serum (Peehl and Stanbridge, 1981). Since serum provides growth factors, it is hypothesized that cells with the ability to exhibit anchorage independent growth in medium containing a low concentration of serum are able to synthesize the needed growth factors or are able to replicate without them. This suggestion is supported by the fact that many transformed cell lines are capable of synthesizing growth factors. In an autocrine manner, these growth factors stimulate the proliferation of the cells which produce the growth factor (Kaplan et al., 1982).

In order to understand how retinoids might affect carcinogenesis, their effect on anchorage independent growth of tumor-derived cells has been examined. For example, Meyskens et al. (1983) showed that the anchorage independent growth of 49% of the tumor-derived cell suspensions tested was decreased by retinoic acid and growth of 14% of them was decreased by 4-hydroxyphenyl-all-trans-retinamide. Retinoids have also been reported to inhibit anchorage independent growth of myc-transfected Fischer rat 3T3 cells induced by growth factors (Roberts et al., 1985), but were found to stimulate the anchorage independent growth of normal rat kidney cells (vanZoelen et al., 1986).

The purpose of the research presented in this dissertation was to provide information about the effect of retinoids on anchorage independent growth of human diploid fibroblasts. Chapter 2 is a manuscript describing research to determine which growth factor(s) induce anchorage independent growth of human fibroblasts. This manuscript has been accepted for publication in the Journal of Cellular Physiology. Chapter 3 represents results of experiments designed to determine the effect of retinoids on anchorage independent growth of cells derived from a human fibrosarcoma and of normal human fibroblasts induced to grow in semisolid medium by specific growth factors. This manuscript has been submitted for publication in the International Journal of Cancer.

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Chapter 1

LITERATURE REVIEW

I. The etiology of cancer

Cancer is defined in Stedman's Medical Dictionary as "a general term frequently used to indicate any of various types of malignant neoplasms, most of which invade surrounding tissues, may metastasize to several sites, and are likely to recur after attempted removal..". A neoplasm is defined as "new growth; an abnormal tissue that grows by cellular proliferation more rapidly than normal ..... Neoplasms show partial or complete lack of structural organization and functional coordination with the normal tissue; they usually form a distinct mass of tissue. The term tumor ..... is frequently used synonymously with the word neoplasm."

A. Mechanisms of carcinogenesis

Cancer can develop in many tissues of the body. Normal growth and development of a tissue involves controlled proliferation and differentiation of the component cells. Mechanisms of cancer development are considered to involve aberrations in the process of cellular proliferation or differentiation.

1. The clonal origin of tumors

Analysis of tumors has led to the conclusion that most are clonal in origin i.e. a tumor originates from a single cell. This conclusion comes from several lines of evidence. Chemically-induced tumors in chimeric rodents (a chimeric rodent is created by uniting embryonic tissue from two different strains of animals, each with a unique isozyme pattern) are composed of cells which contain only one isoenzyme pattern rather than two. Finding only one isomeric pattern in tumors from these chimeras indicates that a single cell gave rise to the tumor cell population rather than cells from both parents from which the chimera was formed (Iannoccone et al., 1978; Elbling and Sauermann, 1982; Reddy and Fialkow, 1983; Iannaccone et al., 1987). Cytogenetic studies demonstrate that all cells in many primary tumors share certain common abnormalities in their chromosomes which indicates that all of the cells in the tumor were derived from one cell (Sandberg et al., 1967). Similarly, the immunoglobulin produced by plasma cell tumors has the homogeneity characteristic of a single clone (Fialkow, 1974) and cells from tumors of females that are heterozygous for the sex-linked glucose-6-phosphate dehydrogenase (G6PD) locus exhibit a single G6PD phenotype (Linder and Gartler, 1965).

2. Multistep changes are involved in transformation to malignancy

a. In vivo evidence

In vivo evidence for the involvement of several steps or changes is supported by the work of Peto et al. (1975). These investigators determined that the increase in cancer incidence that is observed with age

is related to the time required for the cellular changes involved in carcinogenesis and is not a phenomenon associated with ageing per se. Their conclusion was based on an experiment in which skin cancer was induced in a large group of mice with benzo(a)pyrene applications beginning at 10, 25, 40, or 55 weeks of age. The incidence rate of malignant epithelial tumors was independent of the age of the animals at the start of exposure. The increased incidence was identical in all groups and correlated with the length of exposure. The investigators compared these data to the observed increased incidence rates of most human adult cancer with age and suggested that the duration of the exposure of humans to background and spontaneous carcinogenic stimuli is an important factor in the etiology of cancer.

Early evidence for the involvement of multiple steps in carcinogenesis was published by Berenblum (1940), Mottram (1944), and Berenblum and Shubik (1947). In Berenblum's investigations of skin cancer in mice, he found that initial exposures to non-tumorigenic doses of the carcinogen benzo(a)pyrene, followed by repeated exposure to croton oil, which he showed was non-carcinogenic by itself, were necessary for the development of tumors (papillomas). His interpretation of these results was that skin carcinogenesis is a two stage process. The first stage, referred to as initiation, appeared to result from a permanent, heritable change in a cell. The second stage, during which an initiated cell gives rise to a benign tumor, is referred to as promotion. During the promotion period, cell proliferation occurs and allows the clonal expansion of the altered cell (Potter, 1981). This stage has been shown to be reversible or nonpermanent (Boutwell, 1964). The two-stage model has provided a

framework for investigation of other types of cancer including liver cancer (Pitot and Sirica, 1980).

A third stage in tumorigenesis, progression, is defined as the development of malignancy by way of a permanent irreversible qualitative change in one or more of the tumor's characters leading to increased autonomy (Foulds, 1957). The permanent nature of the change associated with progression was shown by Hennings et al. (1983). The majority of tumors that develop in mouse skin with the initiation-promotion protocol are papillomas, a benign tumor of the skin. Conversion of papillomas to carcinomas was shown to occur as a result of treatment with an initiating compound (Hennings et al., 1983). After the promotion phase of the experiment, the type of compound that functioned as an agent for progression was tested. Animals treated with initating compounds (N-methyl-N'-mitro-N-nitrosoguanidine, MNNG or 4-nitroquinoline-N-oxide applied to the skin or urethane administered by intraperitoneal injections) developed more carcinomas than the groups treated with either the promoter, 12-O-tetradecanoylphorbol-13acetate (TPA), or the solvent control. This supports the idea that multiple, permanent changes are involved in the progression of a tumor to malignancy.

b. <u>In vitro</u> evidence

Support for the multistage model of carcinogenesis is also found in experiments with cells in culture. Phenotypic markers that are commonly associated with transformation of cells in culture are immortality, anchorage independent growth (Land et al., 1983) and growth factor independence (Perez-Rodriguez, 1981). Anchorage independent growth (the

ability to form colonies in semisolid medium) is a marker that is highly correlated with tumorigenicity of mouse fibroblasts in athymic mice (Freedman and Shin, 1974; Shin et al., 1975). Milo et al. (1981) have reported that anchorage independence of chemically transformed human fibroblasts also correlated with the formation of nodules in athymic mice. Cifone and Fidler (1980) showed that the pattern of anchorage independent growth, i.e. the rate of development and the size of the colonies, in mouse cells is related to the metastatic potential of the cells.

A series of experiments reported by Pouyssegur and his colleagues further substantiate the requirement for a series of changes in the progression of cellular transformation to a malignant phenotype. For example, Perez-Rodriguez et al. (1981) studied a Chinese hamster fibroblast cell line, CCL39, that was derived from secondary cultures of adult fibroblast cells and exhibited an infinite lifespan. These cells were anchorage dependent and required serum for growth. Within 5-6 weeks after injection of the CCL39 cells into athymic mice, palpable nodules developed. The cells derived from the tumors had different properties from those injected into the mice. The tumor-derived cells were anchorage independent and independent of the requirement for serum growth factors. The authors concluded that an _in vivo_ selection was necessary for CCL39 cells to spontaneously acquire the malignant phenotype.

In another experiment, they mutagenized the CCL39 cells with ethyl methane sulfonate and selected them for anchorage independence. When they analyzed cells from 15 anchorage independent colonies for growth factor independence, they found no correlation between the ability of cells to form colonies in agarose and their independence from the serum-growth

factor requirement for DNA synthesis. Progeny cells from each of the anchorage independent clones were injected into athymic mice. Cells from the tumors that developed were analyzed for their growth factor requirements. Comparison of the serum requirements of the tumor-derived cells to those of the corresponding anchorage independent cells that were injected showed that all of the tumor-derived cells, with the exception of one, had acquired, in vivo, a stable phenotype allowing for partial or total escape from a growth factor requirement. The one set of cells that was an exception had already acquired growth factor independence prior to injection into the athymic mice. These investigators interpreted their results as indicating that the partial loss of the growth factor requirement in vivo is an essential character for bypassing the growth restraints imposed by the host upon CCL39 cells.

B. Theories on the origin of cellular changes required for carcinogenesis

1. The epigenetic theory

Two theories that have been postulated for the origins of cellular changes are the epigenetic theory and the genetic theory. The epigenetic theory classifies cancer as a disease in which cells fail to differentiate normally. The mechanisms of carcinogenesis, according to the epigenetic theory, involve alterations in the normal programming of the cell that do not involve permanent changes in the genome (Markert, 1968). The concept that the origin of cancer is epigenetic and involves a developmental basis (reviewed by Rubin, 1985) is supported by the fact that disruption of normal topographical tissue relationships result in cancer. An example of this was reported by Biskind and Biskind (1944), who showed that

transplantation of rat testis to the spleen resulted in interstitial cell tumors. Further evidence for the epigenetic theory as a basis for the origin of cellular changes leading to malignancy is the fact that teratocarcinoma-derived cells developed into normal tissues after injection into mouse blastocysts (Mintz and Illmensee, 1975). The development of normal tissue from these cells indicates that the genome was not altered during development of the teratocarcinoma. The mouse teratocarcinoma cells, derived from a tumor that developed after transplantation of a 6-day male 129/Sv Sl^{J} C P agouti black mouse embryo underneath the testis capsule, were cultured in vivo by intraperitoneal transfers in syngeneic males for 200 transplant generations (eight years). These cells, cultured in vivo, maintained a normal diploid chromosome complement. The teratocarcinoma cells were isolated and injected into blastocysts from the non-agouti brown C57-b/b strain of mice. The blastocysts were transferred to a pseudopregnant mouse. Some of the mice that were born had hair color and/or internal tissues of the 129 tumor-strain. It is not apparent how normal development of tissues from the teratocarcinoma cells would have been possible if changes in the genome had been responsible for the cellular changes that lead to a tumor.

2. The genetic theory

a. In vivo evidence

Although there is strong evidence for the epigenetic theory as an explanation for the origin of some cancers, there is stronger evidence to support the genetic theory as a valid explanation for the origin of the cellular changes in carcinogenesis. The genetic theory attributes the

origin of the changes in a cell to a change in the DNA (Cairns, 1981; Bishop, 1987). A permanent change in the DNA that results in a selective growth advantage for a cell will enable survival, transmission of the change, and a proliferative capacity that results in the altered cells outnumbering the normal cells in the population. Examples of the kinds of changes in DNA that can result in permanent changes in gene expression are mutations (i.e. base pair substitutions or deletions), chromosomal rearrangements, and amplification (an increased number of copies of a gene). There are many examples that support the idea that changes in the genome may be involved in the development of cancer. Rowley (1983, 1984) and Yunis (1983) have shown that some types of human leukemia and lymphoma cancers contain specific structural aberrations of chromosomes. Yunis (1983) also indicated that deletion of a specific chromosome band is common in carcinomas. Other evidence includes the fact that predisposition for some types of cancer is hereditary (Knudson, 1985; Li, 1988); people who have an impaired ability to repair DNA have an increased susceptibility to cancer (Cleaver, 1968); and many environmental agents associated with carcinogenesis are mutagens (Ames, 1979).

b. <u>In vitro</u> evidence

Evidence showing that genetic changes are involved in the malignant transformation also comes from experiments using cells in culture. Using chemical carcinogen treated Syrian hamster embryo cells, Barrett and Ts'o, (1978), determined the frequency of somatic mutations at the Na^+/K^+ ATPase and HPRT locus and the frequency of transformation determined by anchorage independent growth. The frequency of anchorage independent growth was

similar to the frequency of somatic mutation; however, the time of expression was much longer than the expression time for a somatic mutation. In the light of more recent findings, these investigators now consider that the exposure of the cells to carcinogen resulted in some of the cells acquiring an infinite lifespan in culture, and the anchorage independence was a result of a second, spontaneous mutation that occurred during culturing of the chemically-treated cells (personal communication, P.T.). This conclusion is supported by the results of Newbold et al. (1982) who reported that treatment of Syrian hamster cell cultures with carcinogenic chemicals can induce rare immortal variants, the progeny of which frequently progress to anchorage independence and malignancy after a further period of growth. Similar to the above experiments, Spandidos and Siminovitch (1978) compared the frequency of transformation, measured by anchorage independent growth, induced by benzo(a)pyrene or urethane in Chinese hamster fibroblasts to the frequency of somatic mutations measured by resistance to thioguanine or ouabain. The results obtained were similar to those obtained by Barrett and Ts'o (1978). The anchorage independent phenotype appeared after 2 months of culturing the cells and the frequency was similar to the induced mutation frequency. They also tested the tumorigenicity of the cells and found that it was associated with the ability of the cells to grow in anchorage independent conditions. It has been well documented that human fibroblast cells can be induced by carcinogen treatment to acquire anchorage independence as an early phenotype at frequencies comparable to the frequency of induced mutations (Sutherland et al., 1980; Silinskas et al., 1981). However, these cells do not acquire an infinite lifespan and are not malignant. This is in

contrast to rodent fibroblasts which can become malignantly transformed. The spontaneous acquisition of an infinite lifespan in Chinese hamster cell cultures occurs at a rate that would be expected for a spontaneous mutation (Kraemer et al., 1986); whereas the infinite lifespan phenotype is not normally acquired by human fibroblast cell cultures. Therefore, the rareness of acqiring an infinite lifespan, a step in the transformation process, may be the reason that human fibroblasts are rarely transformed to malignancy in culture.

There is excellent evidence that a permanent change in the genome can be responsible for the origin of the cellular changes seen in malignant cells. This implies that specific, critical genes involved in growth and/or differentiation are the sites of changes in the DNA during carcinogenesis. Current research on the involvement of cellular genes (proto-oncogenes) and their altered counterparts (oncogenes) in cancer will be discussed in section II.

II. The role of peptide growth factors and oncogenes in carcinogenesis

A. Introduction

1. Peptide growth factors

Peptide growth factors are able to control cell proliferation by their ability to stimulate or inhibit DNA synthesis and cell division. Some of these growth factors, such as nerve growth factor (Levi-Montalcini and Angeletti, 1968), act on a specific cell type. Others, such as platelet-derived growth factor, (Antoniades et al., 1985), act on cells of similar embryonic origin. Still others, such as epidermal growth factor (Carpenter and Cohen, 1979) are able to have an affect on a broad range of cell types. A peptide growth factor may affect target cells by several mechanisms. These include endocrine (i.e. the growth factor is transported through the bloodstream to act on target cells distal to the site of synthesis), paracrine (i.e. it affects cells in close proximity to the site of synthesis) or autocrine (the target cell is the same cell that synthesized the growth factor) mechanisms (Sporn and Todaro, 1980).

Peptide growth factors function by binding to a specific receptor on the cell surface. This binding initiates a cascade of changes that lead to DNA synthesis and cell proliferation. Several pathways exist to transmit the message to start DNA replication. As shown in Figure 1, the binding of the growth factor to its receptor stimulates protein kinase activity of the receptor itself, or protein kinases in the membrane or cytosol of the cell. Unlike the protein kinases involved in other cellular reactions, these enzymes phosphorylate tyrosine in the protein substrate (Cooper et al., 1982). It is not entirely clear how the message to replicate DNA is transmitted to the nucleus. However, after growth

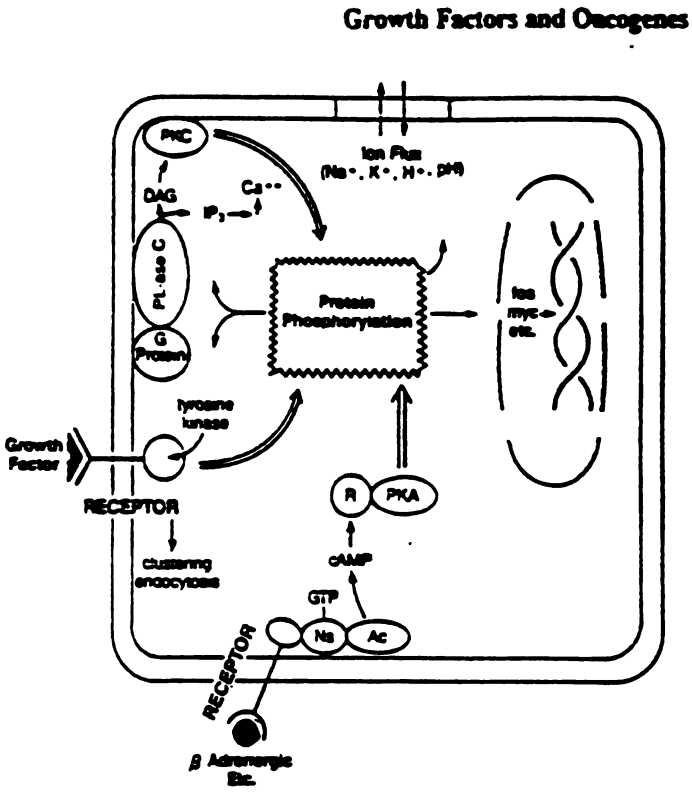

Fig. 1. A schematic diagram of a cell showing various pathways of membrane-associated receptors and signal transduction. The growth factor pathway applies to EGF, PDGF, and insulin. The beta-adrenergic pathway involves coupling via a G regulatory protein (Ns) to adenyl cyclase (Ac), and cyclic AMP (cAMP) binding to the regulatory subunit (R) of protein kinase A (PKA). Various agonists can activate phospholipase C (PL-ase C), presumably via a G protein, leading to the release of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG activates protein kinase (PKC) and IP_3 causes the release of Ca^{++} from the endoplasmic reticulum. These events lead to a cascade of protein phosphorylation that alters the functions of membrane-associated receptors, ion channels, and cytoplasmic proteins. Signals (undefined) also enter the nucleus to induce the expression of various genes including *c-fos* and *c-myc*.
Taken from J. Cell Biochem. 33:213-224, with permission of Alan R. Liss Inc.

factor binding, transient transcription of specific genes does occur (Muller et al., 1984).

Examples of growth factors that have different pathways for transmission of signals leading to DNA synthesis are PDGF and EGF. The binding of PDGF to its receptor results in the stimulation of phospholipase C and the hydrolysis of phosphatidylinositol bisphosphate in the membrane which results in the release of diacylglycerol and 1,4,5 inositol triphosphate (Habinicht et al., 1981; Berridge et al., 1984). Diacylglycerol stimulates membrane protein kinase C activity, while 1,4,5 inositol triphosphate stimulates an increase in intracellular calcium concentration. EGF is similar to PDGF in that it binds to a specific membrane receptor. The EGF receptor, however, has protein kinase activity associated with the cytoplasmic domain. This kinase activity is stimulated by ligand binding to the receptor. The receptor protein kinase can autophosphorylate the receptor or phosphorylate tyrosine residues of specific cytoplasmic proteins. Shortly after the phosphorylation of these proteins, transcription of <u>myc</u> and <u>fos</u> genes is observed (Muller et al., 1984).

Since uncontrolled growth is an important characteristic of cancer cells, it is reasonable to consider alterations that result in increased or untimely stimulation of DNA synthesis and cell division as mechanisms of carcinogenesis (Heldin and Westermark, 1984). Possible alterations in a cell include the ability to synthesize a growth factor that will stimulate the growth of the cell, the ability to synthesize a growth factor receptor, or the ability to increase the signal transmission for DNA synthesis by constituitive activation of the signal. An example of

cells that acquire the ability to synthesize a growth factor is found in the work of Bowen-Pope et al. (1984). They reported production of platelet-derived growth factor-like molecules by a variety of cells upon chemical or viral transformation. Another example is the production of growth factors by mouse fibroblasts upon infection with Moloney murine sarcoma virus (DeLarco and Todaro, 1978). These growth factors caused the expression of anchorage independence, a transformed phenotype, and stimulated the cells to divide in monolayer cultures.

2. Oncogenes

Oncogenes can be defined as a class of genes with an ability to confer a transformed phenotype on cells in tissue culture and contribute to the tumorigenicity of the cells in vivo (Heldin et al., 1987). The first oncogene was identified as the single gene responsible for the tumorigenicity of Rous sarcoma virus and is referred to as src (Hanafusa and Hanafusa, 1966). The fact that the cellular genome could be the source of the retroviral transforming DNA was first reported by Stehelin et al. (1976). They discovered that the transforming sequences of the avian sarcoma virus were homologous to cellular DNA sequences of several avian species but not mammalian species. From this it has been hypothesized that retroviral transforming sequences are transduced or acquired from the host genome. If this acquired sequence confers an advantage to the host cell for survival, the transduced viral sequence is conserved in nature. Since this recognition, an increasing understanding of the involvement of oncogenes in carcinogenesis is developing (Varmus, 1984; Bishop, 1987; Guerrero and Pellicer, 1987; Spandidos and Anderson,

1987).

The cellular genes that have sequence homology with the retroviral oncogenes are referred to as proto-oncogenes. The term oncogene was at first associated only with the transforming gene of acutely transforming retroviruses. It is now recognized that specific genetic alterations occur in tumors. The term oncogene now refers to the activated form of the cellular gene that is found in tumors. Activation can occur when the cellular gene is mutated (Guerrero and Pellicer, 1987), rearranged (Shen-Ong et al., 1982), or amplified (Quintanilla et al., 1986). Amplification of the gene product can also occur when the regulatory region for transcription of the gene becomes mutated.

Oncogenes have been classified according to their cellular location: nuclear or cytoplasmic (Weinberg, 1985) or according to their known functions: tyrosine protein kinase activity, growth factor, GTP binding proteins, or nuclear proteins (Hunter, 1984). More than 40 oncogenes have been identified. Oncogenes have often been identified by the ability to transform recipient cells, such as NIH 3T3 fibroblasts, in a gene transfer experiment (Cooper, 1982).

B. Proto-oncogenes and oncogenes function as growth factors

Autocrine synthesis of growth factors (DeLarco and Todaro, 1978) and loss of a requirement for epidermal growth factor (Cherington et al., 1979) have been associated with transformation of cells. These observations support the possibility that proto-oncogenes and oncogenes which code for growth factors are involved in cellular transformation. An example of this is the v-sis oncogene product of the Simian sarcoma

virus (SSV) which has been identified to be homologous with the B-chain of PDGF (Doolittle et al., 1983). The v-sis product of an SSV-transformed cell is dimerized and processed resulting in a 24 kD dimer homologous to a homodimer of PDGF B-chains (Robbins et al., 1983). The functional properties of the v-sis and c-sis gene products appear to be similar (Johnsson et al., 1985). Transfection of human fibroblasts with v-sis transforms the cells morphologically, induces foci formation, and induces anchorage independence, but does not confer tumorigenicity on the cells (Fry et al., 1986). Stevens et al., (1988) reported similar effects after transfection of c-sis cDNA in an expression vector into human fibroblasts. Cloned c-sis cDNA and genomic sequences placed under strong viral promoter can also induce transformation when transfected into NIH 3T3 cells (Clarke et al., 1984). Further support of the involvement of the sis gene in carcinogenesis is that c-sis is expressed by some human tumor cell lines but not by normal cells (Betsholtz et al., 1986). Another growth factor, transforming growth factor-α, is produced by cells transformed by certain retroviruses (DeLarco and Todaro, 1978; Ozanne et al., 1980) and by many cell lines established from tumors (Todaro et al., 1980).

C. Proto-oncogenes and oncogenes function as receptors

Since growth factor binding to a receptor stimulates protein kinase activity of the receptor or cellular kinases, an increased number of receptors or increased receptor kinase activity would be expected to increase cell proliferation. An example of this is the work of DiFiore et al. (1987) who reported the transformation of NIH 3T3 cells after transfection with EGF receptor cDNA under the transcriptional control of

the Moloney murine leukemia virus LTR, a strong promoter. When EGF was added to the medium, the transfected cells formed foci, which is characteristic of tumor-derived cells. Further evidence of the transformation was the high frequency of anchorage independent growth of the EGF receptor-transfected NIH 3T3 cells compared to appropriate controls. This overexpression of the EGF receptor in NIH-3T3 cells was similar to the overexpression seen in human tumor cell lines that exhibit EGF receptor gene amplification (Lin et al., 1984; Ullrich et al., 1984; Kraus et al., 1987).

Another gene related to a growth factor receptor is the oncogene, v-erb-B, that codes for the transforming protein from the avian erythroblastosis virus. The gene product has 95% homology with the human EGF receptor and resembles a truncated EGF receptor that is missing the binding domain for the growth factor and a portion of the cytoplasmic carboxy-terminus end which has kinase activity (Ullrich et al., 1984). The loss of regulation that occurs with these two deletions has been postulated to result in constituitive activation of the cellular kinase domain which could account for the ability of v-erb B to transform fibroblasts (Ng and Privalsky, 1986). Other oncogene products have been identified with structures indicating that the normal gene product is a growth factor receptor. These include c-fms, colony-stimulating factor-1 receptor (Sherr et al., 1985); neu, related to the EGF receptor, (Schechter et al., 1984); v-ros, related to the insulin receptor (Neckameyer et al., 1986) and v-met, related to the insulin receptor (Dean et al., 1985).

D. Proto-oncogenes and oncogenes function in the growth factor signal pathway: regulatory proteins associated with the cell membrane

The binding of a peptide growth factor to specific receptors on the cell membrane stimulates the activity of enzymes in the membrane. One of these enzymes, phospholipase C, appears to be regulated by a GTP-binding protein (Cockcroft and Gomperts, 1985). A family of genes, ras, codes for proteins that also have GDP and GTP-binding properties (Scolnick et al., 1979; Shih et al., 1982) and GTPase activities (McGrath et al., 1984; Gibbs et al., 1984). As discussed above, activation of phospholipase C produces two products, diacylglycerol and 1,4,5 - inositol triphosphate, which stimulate protein kinase C and the release of intracellular stores of calcium into the cytosol as part of the growth factor signal transduction pathway. Although there is little direct data to support a role for ras genes in this pathway, it has been speculated that the loss of GTPase activity in oncogenic ras proteins may cause these proteins to activate phospholipase C in an uncontrolled way (Berridge et al., 1984; Fleischman et al., 1986).

Presently, there are four known members of the ras gene family. One of them was first identified as the transforming genes of the Harvey sarcoma virus and the other was identified as the transforming gene of the Kirsten sarcoma virus. They are referred to as H-ras and K-ras respectively (Ellis et al., 1981). N-ras was first identified in a human neuroblastoma (Shimizu et al., 1983). A fourth ras gene, R-ras, has recently been identified (Lowe et al., 1987). Activation of these genes by either point mutation (Reddy et al., 1982; Land et al., 1983) or amplification (Barbacid, 1987) or both (Hurlin et al., 1987) is associated

with transformation to focus formation, anchorage independence and growth factor independence of diploid fibroblasts. These transformed cells, however, are not fully transformed to cells with the ability to form malignant tumors.

The involvement of ras genes in carcinogenesis is also indicated by the fact that mutated ras genes have been identified in 10% - 20% of human tumors; and even this number may be an underestimation because of limitations of the techniques used for detection. Activation of ras genes has also been identified in many carcinogen-induced tumors in animals (Zarbl et al., 1985; reviewed in Guerrero and Pellicer, 1987).

E. Proto-oncogenes and oncogenes function in the growth factor signal pathway: regulatory proteins in the nucleus

The transcription of several cellular genes is activated after a growth factor binds to the cell membrane (Rittling et al., 1986; Kruijer et al., 1984). A few of these genes, including c-myc (Kelly et al., 1983), c-fos (Greenberg and Ziff, 1984), c-myb (Thompson et al., 1986), and c-jun (Angel et al., 1988), code for proteins that reside in the nucleus. Transient expression of c-fos and c-myc occurs in fibroblasts stimulated by PDGF (Greenberg and Ziff, 1984; Kelly et al., 1983), EGF (Paulsson et al., 1987), or FGF (Muller et al., 1984). The function of the protein products of c-myc and c-fos is unclear; however, an ability to bind to DNA has been demonstrated (Donner et al., 1982; Sambucetti and Curran, 1986) which suggests a regulatory function for these gene products. A regulatory function has also been suggested for the c-jun gene product. This protein complexes with the c-fos protein and can

interact with regulatory sequences of many different genes (Rauscher et al., 1988).

Since the gene products of c-myc, c-fos, and c-jun appear to function in a process involving the genome (i.e. transcription or replication) in response to growth factor stimulation of DNA synthesis, it is possible that constituitive expression or overexpression of these genes could increase cellular proliferation. In fact, Armelin et al., (1984) have reported stimulation of the clonal growth of BALB/c-3T3 cells in PDGF-free medium by expression of a transfected c-myc gene under the control of a strong viral promoter. Further evidence for a role of the c-myc gene in transformation is the association of the gene with establishment of cells in culture (infinite lifespan) (Land et al., 1983; Mougneau et al., 1984). Overexpression of c-myc was also associated with anchorage independent growth of a rat fibroblast line transfected with a human c-myc gene under the control of a strong viral promoter (Pellegrini and Basilico, 1986). Some of the progeny of the agar colonies (those that had a refractile morphology) expressed high levels of myc RNA and were tumorigenic in syngeneic rats. Similar results were reported when a high expression vector containing the human c-myc gene was transfected into rat embryo fibroblasts (Land et al., 1986). A small subset of these cells (from clones with a refractile morphology) were tumorigenic.

F. Role of oncogenes and proto-oncogenes in multistep carcinogenesis

Cellular transformation has provided insights into the role of different genes in tumorigenesis. Complementation of genes from two classes is necessary for transformation of NIH 3T3 cells (Land et al.,

1983; Ruley, 1983). The ability to proliferate continuously (establishment), the ability to exhibit anchorage independent growth, and the ability to grow in the presence of reduced concentrations of growth factors must be acquired in order for a cell to become tumorigenic. The myc gene confers establishment (Land et al., 1983; Land et al, 1986); while an activated ras gene confers the properties of anchorage independence and reduced requirements for growth factors (Land et al., 1983; Hurlin et al., 1987; 1988). Another example of the involvement of ras in multistep carcinogenesis is documented in a report by Vogelstein et al. (1988) on the development of colorectal tumors. Histological classification of the tumors into four stages of progression from class I adenomas to carcinomas and the analysis of genetic alterations in the tumors indicated that common sequential changes involving three different allelic deletions and a ras mutation were involved in the etiology of the malignancy.

III. Evidence for the reduction of cancer by retinoids

A. Epidemiologic evidence

Epidemiologic studies provide supportive evidence to indicate that increased consumption of vitamin A is associated with decreased risk for many types of cancer (reviewed by Bertram et al., 1987). These include lung (Bjelke, 1974; Mettlin et al., 1979; Kolonel, 1985; Humble et al., 1987), larynx (Graham et al., 1981), esophagus (Ziegler, et al., 1981), stomach (Stehr et al., 1985), bladder (Mettlin and Graham, 1979), breast (Graham et al., 1982), and cervix (Wylie-Rosett et al., 1984). However, some studies have found no relationship between the level of vitamin A consumption and the risk of lung cancer (Ziegler et al., 1984; Paganini-Hill et al., 1985). Other studies have reported that an increased incidence of some types of cancer is associated with a increased consumption of vitamin A. For example, Hirayama (1979) reported that an increased risk of prostrate cancer was associated with increased consumption of vitamin A.

In an epidemiological survey, the amount of vitamin A that is eaten is often evaluated by the frequency of consumption of food items such as green and yellow vegetables. These foods are a source of preformed vitamin A and also a rich source of carotenoids, precursors of vitamin A. Carotenoids can also be transported and stored as carotenoids and may have an effect on the incidence of cancer independent of the effect of vitamin A (Peto et al., 1981; Machlin and Bendich, 1987). It is, therefore, difficult to evaluate the association of vitamin A consumption with the risk of cancer in many epidemiologic studies. One report that analyzed the association of β-carotene consumption separately from retinol

consumption found that men with the highest β-carotene intake had a lower risk of lung cancer than those with the lowest intake of β-carotene (Ziegler et al., 1984). In the same study, the risk of lung cancer was similar for men who comsumed high or low amounts of retinol. Another study compared the association of dietary retinol, dietary carotene, serum retinol or serum carotene with the risk of lung cancer in women (Pastorino et al., 1987). The consumption of either low levels of carotenoids or low levels of retinol was associated with an increased risk of cancer. The group with the highest serum carotene levels had the lowest risk of lung cancer. Serum retinol concentrations were not associated with cancer risk.

Since plasma retinol is not a good indicator of vitamin A status except in cases of deficiency or excess (Olson, 1984) and since changes in vitamin A consumption within the normal range may affect the risk of cancer, it is unlikely that plasma retinol levels will correlate with the risk of cancer. Kark et al. (1981) first reported a strong association between lower serum retinol concentrations and incidence of lung cancer. A study of the surviving cohort in the same community seven years later showed that serum retinol levels, analyzed by more refined techniques, did not show any relationship to the risk of lung cancer (Peleg et al., 1984).

B. Animal experiments

In general, vitamin A inhibits or retards the development of cancer in animals (Moon et al., 1983; Moon and Itri, 1984). A closer analysis of the literature shows that there is marked variability in the response. The type of cancer, the animal species, and/or the chemical form of the

retinoid can influence the experimental effect of a retinoid on tumor development.

Topical application of retinoids have been found to inhibit skin cancer induced by a two stage, chemical carcinogen-induced model in mice (Bollag, 1971; Verma and Boutwell, 1977; Verma et al., 1979; Weeks et al., 1979; Verma, 1987b). In the first stage, a chemical carcinogen (7,12-dimethylbenz[a]anthracene, DMBA) is applied to the backs of the mice as an "initiator". In the second stage, 12-O-tetradecanoylphorbol-13-acetate (TPA) is applied to the backs of the mice several times a week for several weeks (20 - 30) as a "promoter". The retinoid is applied with the TPA during the promotion phase. In contrast to the ability of retinoids to retard development of TPA-promoted papillomas of the skin, retinoic acid can act as a promoter in the absence of TPA (Hennings et al., 1982; McCormick et al., 1987).

Experimental mammary cancer is also reduced by retinoid administration. Moon et. al. (1983) reviewed data from experiments which tested several retinoids for their ability to modify rat mammary carcinogenesis initiated by N-methyl-N-nitrosourea (MNU). Retinyl acetate, 13-cis- retinoic acid, and retinyl methyl ether were effective in reducing the mammary cancer; however, isotretenoin (13-cis-retinoic acid) and several synthetic retinoids were not effective. The retinoids were administered during the promotion phase of the experimentally induced cancer. Even when the administration of retinyl acetate was delayed until 4 weeks after rats were initiated with a high dose of MNU or until 12 weeks after rats were initiated with a low dose of the carcinogen, a decrease in the cumulative tumors per rat was observed (McCormick and

Moon, 1982). As in skin cancer, retinoids exhibit an inhibitory effect during the promotional phase of carcinogenesis (Welsch et.al., 1985). Treatment with retinoids during initiation has also resulted in a decrease in rat mammary cancer (McCormick et al., 1980; 1981).

In each of the previous studies, pharmacological amounts (100 - 300 mg per day) of retinoids were administered. Zile et al. (1986) tested the effect of more physiologic levels of dietary retinyl acetate (3 - 30 ug/day) on the incidence of mammary tumors initiated with a low dose of DMBA. The low dose resulted in a decreased number of carcinomas and an increased number of benign mammary neoplasms over a longer time compared to the tumor development that occurs with higher doses of DMBA. When data from experimental groups were pooled to increase the number of animals per group (100 - 150), the total number of mammary tumors was significantly decreased in rats receiving either marginal or moderately elevated intakes of retinyl acetate compared to rats fed an adequate daily intake. These differences were found during both the initiation period and the promotion period. Others have reported that a deficiency of vitamin A increases the susceptibility to certain epithelial cancers (Davies, 1967; Rogers et al., 1973). In contrast to the majority of reports that administration of retinoids retard experimental mammary cancer, retinyl acetate enhanced mammary cancer in GR/A mice (Welsch et al., 1981).

Retinoids also decrease bladder cancer induced in rodents by MNU or N-butyl-N-(4-hydroxybutyl)nitrosamine (Grubbs et.al., 1977, Sporn et al., 1977). The results from these experiments showed that the retinoids that were effective or ineffective in inhibiting bladder cancer did not necessarily have the same effect on mammary cancer. For example, 13-cis-

retinoic acid did not inhibit development of mammary cancer, but inhibited bladder cancer (Moon et al., 1983).

The effect of retinoids on cancer of other sites has also been studied. Rat bronchial cancer induced by 3-methylcholanthrene is inhibited by retinyl acetate (Cone and Nettesheim, 1973; Nettesheim et al., 1976); but respiratory tract tumors induced by benz(a)pyrene in hamsters increased when the animals were treated with retinyl acetate (Smith et al, 1975). Four different synthetic retinoids were shown to inhibit azaserine-induced pancreatic cancer in rats (Longnecker et al., 1983). However, azaserine-induced neoplasms also developed at many other sites, including liver, kidney, skin and breast. The incidence of neoplasms at these other sites, except for the liver, was similar between the control and retinoid treated animals. Treatment with three of the retinoids resulted in an increased incidence of carcinomas and neoplastic nodules in the liver of the female rats. No differences were found in the male rats. An increase in the size of carcinomas and the degree of malignancy in tumors of the hamster cheek pouch was observed when topical retinyl palmitate was applied after induction with DMBA (Polliack and Levij, 1969).

These animal experiments indicate that retinoids, in general, decrease the frequency and size of tumors, but may increase the development of some types of cancer in different animal model systems. A better understanding of the mechanism of retinoid action could provide insights into how retinoids can be used effectively in the prevention and/or treatment of human cancer.

C. Clinical Studies

Vitamin A has been used clinically for a variety of skin disorders. The similarity of the vitamin A deficiency symptom of follicular keratosis of the skin with some treatment-resistant skin disorders such as Darier's disease and pityriasis rubra pilaris. Initially, tretinoin (all-trans-retinoic acid) was used to treat these skin disorders. In 1976, the less toxic isotretinoin (13-cis-retinoic acid) became available for clinical testing in the U.S. and etretinate (an aromatic analogue of retinoic acid) became available for clinical trials in 1978. Both of the synthetic retinoids, isotretinoin and etretinate, were more effective and less toxic than vitamin A (Mayer et al., 1978; Peck, 1980).

Isotretinoin has also been successfully used to treat skin cancer (Peck et al.,1983). Eleven patients with multiple basal cell carcinomas were given an average maximum oral dose of 4.7 mg/kg/day of isotretinoin. Of 248 total tumors, 16% completely regressed, 65% partially regressed, and 19% were unchanged (Peck, 1979). In 1982, the results of long term treatment of 3 of the original 11 patients were reported (Peck, 1982). Treatment with 1.5 mg/kg/day of isotretinoin resulted in complete regression of 9 of 65 tumors. Most noteable was the fact that no new tumors were observed in any of the patients. A follow-up of two of the above patients after 7 or 8 years of treatment was reported (Peck, 1978). Initially, these patients received a high dose of isotretinoin (2.0 - 3.0 mg/kg/day) as chemotherapeutic treatment. After one year, the dose was reduced to 1.5 and then progressively reduced to 0.25 mg/kg/day to achieve chemoprevention. One of the patients developed 1 new lesion during therapy with 1.0 mg/kg/day, another lesion with therapy of 0.5 mg/kg/day

and 5 new lesions during treatment with 0.25 mg/kg/day. When treatment was discontinued, the patient developed 29 new tumors within 13 months. The other patient developed his first new tumor 17 months after discontinuing therapy. These findings suggest that long-term therapy with isotretinoin is necessary for continuation of a chemoprotective effect.

The effectiveness of oral isotretinoin on skin cancer was also demonstrated in a three year controlled prospective study on 5 patients with xeroderma pigmentosum and a history of multiple cutaneous basal-cell or squamous-cell carcinomas (Kraemer et al., 1988). Xeroderma pigmentosum is a rare, autosomal recessive disorder with an impaired ability to repair ultraviolet-damaged DNA. The patients are sensitive to sunlight and have a high frequency of sunlight induced skin cancers. During the first 2 years prior to the study, retinoid treatment was not given. Records of the patients prior to referral were obtained from the referring physician. After referral, patients were given complete examinations by at least two of the dermatologists conducting the study. Unusual skin lesion were photographed to document the date of appearance. Any histologically comfirmed skin cancers were surgically removed. Oral treatment with isotretinoin was taken for 2 years followed by a 1 year follow-up during which there was no treatment. Prior to treatment, 121 tumors were surgically removed from the five patients. During the 2 years of treatment, 25 tumors occured. During the last year of the study, without isotretinoin therapy, 81 tumors were removed. While the treatment appeared to be successful some side effects were seen. Another study has reported successful use of isotretinoin in the treatment of refractory squamous cell carcinoma of the skin (Lippman and Meyskens, 1987).

Clinical trials with other premalignant or neoplastic lesions have been conducted (Bollag and Hartmann, 1983). Reports on retinoids tested for treatment of bronchial metaplasias (Gouveia et al.,1982) and recurrent superficial bladder tumors (Alfthan et al., 1983) show potential usefulness of retinoids for chemoprevention of cancer at sites other than the skin.

All of the experimental reports on the effect of oral administration of a retinoid on cancer that are reviewed here, except one (Zile et al., 1986), employed pharmacologic amounts of the retinoid. The ability of amounts of vitamin A required for normal nutrition to have an impact on cancer is less clear. The more physiological amounts of vitamin A tested by Zile et al. (1986) were marginally effective in reducing the incidence of mammary cancer.

IV. Effect of retinoids on cell growth and cellular transformation

A. Introduction

Since retinoids retard the growth of tumors, it was thought that they might act by selectively inhibiting the growth of transformed cells. From studies on growth of cells in culture, it is not easy to generalize about the effect of retinoids on growth (reviewed by Lotan, 1980). High concentrations of retinoids (100 uM retinol; 1-10 uM retinoic acid) are usually toxic to cells in culture. At non-cytotoxic concentrations, treatment with retinoids inhibits the growth of some cells. However, not all transformed cells are inhibited by treatment with a retinoid; nor is the effect of growth inhibition limited to transformed cells. Growth of some cells in culture, such as established mouse fibroblast cell lines 3T3, 3T6 (Jetten et al., 1979) and L-928 (Dion et al., 1977) are inhibited by retinoic acid (10 uM, which was reported as non-cytotoxic to these cells). Inconsistent results have been observed when human fibroblasts were treated with retinoids. Lacroix et al. (1980) reported that the growth of three of four cell strains was inhibited by 10 uM retinoic acid, while Harper and Savage (1980) reported that 0.3 -30 uM 13-cis retinoic acid or all-trans retinoic acid did not affect the growth of human fibroblasts. Lotan and Nicolson (1977) reported a range of sensitivities to growth inhibition by retinoids on untransformed, transformed or tumor cell lines. Many of these investigators have tested the effect of retinoid concentrations that were higher than normal, physiologic concentrations. In the blood, physiologic concentrationsof retinol are in the uM range; while, physiologic concentrations of retinoic acid are nM quantities.

Retinoids have been shown to inhibit the expression of transformation of cells in culture. Extensive studies by Merriman and Bertram (1979) and Bertram (1980) reported that retinyl acetate and other retinoids suppressed chemical carcinogen-induced transformation, measured by the formation of foci, of C3H/10T1/2 cells. The effective concentrations of retinol (approximately 2 uM) and retinyl acetate (approximately 2 uM) are in the physiologically normal range for humans. The effect was observed even when retinoid treatment was not begun until three weeks after carcinogen treatment. The inhibition of transformation was reversible i.e. when retinyl acetate was removed from the media, transformed foci developed. Anchorage independent growth, a phenotype acquired in the process of transformation of fibroblasts to malignant cells, can be inhibited by retinoids. Dion et al. (1978), Lotan et al. (1982), Mukherjee et al. (1983) and Meyskens et al. (1983) have reported the inhibition of several virally or chemically transformed cells as well as tumor-derived cells from rodents and humans. The effective concentration of retinoic acid for most of the cell lines tested was 1 -10 uM, a non-physiologic amount. In contrast to this inhibitory effect, retinoic acid (1 uM or 0.2 uM) increases the anchorage independent growth of normal rat kidney cells (NRK) (Jetten and Goldfarb, 1983; van Zoelen, 1986). However, for some transformed cells, the same concentration of retinoic acid (10 uM) decreases anchorage independent growth, but does not affect monolayer growth (Dion et al., 1977; Dion et al., 1978; Roberts and Sporn, 1984) indicating that retinoids can have a specific effect on this transformed phenotype.

B. General mechanisms for the function of retinoids in growth and differentiation

1. Description of the mechanisms

Two general mechanisms have been postulated for the physiological function of the retinoids known as vitamin A in growth and differentiation. One mechanism that is postulated is that vitamin A is transported into the nucleus and functions by regulating gene expression. The other mechanism hypothesizes that vitamin A functions as a mannose doner in the synthesis of glycoconjugates.

The presence of specific cellular binding proteins for the active forms of vitamin A provides evidence for the function of vitamin A by regulation of gene expression (Chytil and Ong, 1979). These binding proteins are postulated to transport vitamin A to the nucleus. Binding sites on rat liver nuclear membranes have been identified for the cellular retinol-binding protein - retinol complex (Takase et al., 1979). Nuclear binding sites for the complex have also been identified on the chromatin (Liau et al., 1981). The presence of these binding proteins suggest that vitamin A may function like steroid hormones in controlling transcription. Steroid hormones bind to specific cytoplasmic proteins forming a complex which is transported to the nucleus. This complex binds to DNA and regulates gene transcription (O'Malley and Means, 1974). Further support of this mechanism is the identification of a nuclear receptor for retinoic acid (Petkovich et al., 1987; Giguere et al., 1987; Brand et al., 1988).

Vitamin A also functions in the synthesis of glycoconjugates as a carrier of mannose units across the cell membrane (De Luca et al., 1979). Mannosyl retinyl phosphate, a phosphorylated form of vitamin A which provides the mannosyl units for the syunthesis of glycoconjugates, has been identified (DeLuca et al., 1970). It is not clear how this mannose carrier is distinct from dolichyl mannosyl phosphate. However, retinol and retinoic acid have been reported to be directly involved in the biosynthesis of glycoproteins (DeLuca et al., 1975).

2. The ability of retinol and retinoic acid to function in these mechanisms

The physiologically active form of vitamin A that can support growth, as shown by a rat bioassay, is either retinol or retinoic acid (Zile and DeLuca, 1968). Since retinol or retinyl acetate can be metabolized to retinoic acid (Emerick et al., 1967; Kleiner-Bossaler and DeLuca, 1971), it has been suggested that retinoic acid is the active metabolite for growth. The reverse reaction, the reduction of retinoic acid to retinol or retinaldehyde, is not a physiologic reaction (Arens and van Dorp, 1946; Dowlings and Wald, 1960) A number of assay systems have indicated that retinoic acid is more active than retinyl acetate or retinol in controlling differentiation (Sporn and Roberts, 1984).

C. Mechanisms of the effect of retinoids on cellular transformation

1. Retinoids and gene expression

Since retinoids are important in normal growth and differentiation (Fell and Mellanby, 1953; Roberts and Sporn, 1984), it has been suggested

that they may act by controlling transcription of specific genes. This mechanism is supported by the fact that retinoic acid treatment of HL-60 promyelocytic leukemia cells induces differentiation and decreases c-myc transcription (Grosso and Pitot, 1985). Similarily, retinoic acid causes a decreased expression of N-myc which is followed by differentiation in human neuroblastoma (Thiele et al., 1985). However, Dean et al. (1986) reported that the level of c-myc expression was post-transcriptionally regulated and related to growth arrest, but not to differentiation of F9 teratocarcinoma cells after treatment with retinoic acid. The authors speculated that retinoic acid induced or activated specific nuclease activity distinct from the induction of differentiation-specific genes. Gene expression associated with transformation has not been consistently affected by retinoids. For example, Giese et al. (1985) showed that anchorage independent growth of NIH 3T3 cells induced by viral transformation was more sensitive to inhibition by retinoic acid treatment than virally transformed NRK cells. The concentration of retinoic acid that resulted in 50% inhibition of colony formation by the transformed NIH 3T3 cells was 1 -10 nM; the concentration to obtain a similar effect with the virally transformed NRK cells was 1 - 10 uM. The NIH 3T3 cells transformed by murine sarcoma virus which were most sensitive to the effect of retinoic acid on anchorage independent growth, also formed tumors in athymic mice which were sensitive to topical treatment with retinoic acid. However, no difference in the expression of K-ras, v-bas, or v-sis was detected between retinoic acid treated and untreated cells for any of the virally transformed NRK or NIH-3T3 cells. Jetten et al. (1986) observed that anchorage independent growth of v-H-ras transfected

DES-4 cells (clonally derived after treatment of Syrian hamster embryo cells with diethylstilbestrol) was increased by treatment with retinoic acid; whereas, anchorage independent growth of the src-transfected cells was inhibited. A dose dependent effect was observed over a range of retinoic acid concentrations (0.1 uM to 0.1 nM) with a maximum effect at 1 uM. Retinoic acid did not affect the level of $pp60^{src}$ or $p21^{ras}$ proteins, suggesting that the retinoid did not affect gene expression.

2. Retinoids and cytoplasmic binding protein

The presence of cytoplasmic binding proteins for retinol and retinoic acid does not appear to correlate with the ability of the retinoids to have an affect on a cell transformation or cell growth. For example, Mukherjee et al. (1983) did not find any correlation of the presence of these binding proteins with the ability of retinoic acid to inhibit anchorage independent growth. Similarly, Lotan et al. (1980) did not find an association of the presence of cytoplasmic binding proteins with the growth inhibition of neoplastic cell lines by retinoids.

3. Retinoids and regulatory proteins

It has been suggested that retinoids may function by regulating important enzymes involved in cell metabolism. The inhibition of ornithine decarboxylase (ODC, the rate limiting enzyme for the synthesis of spermine) activity by retinoic acid (Verma et al., 1979) and inhibition of transcription of the gene for the enzyme (Verma, 1987a) supports this idea. However, Jetten et al. (1986) reported that retinoic acid treatment of v-H-ras transfected DES-4 cells resulted in increased ODC activity and

increased anchorage independent growth; whereas, decreased ODC activity and decreased anchorage independent growth was seen in v-src transfected DES-4 cells, indicating that the mechanism of retinoid action does not involve ODC activity alone. Another enzymatic step which retinoids have been postulated to control is the phosphorylation of cell proteins. The phosphorylation of low molecular weight membrane proteins (20-30 kD) correlates with the expression of anchorage independent growth by several cell lines (Banerjee et al., 1986). Treatment of some of the cell lines with retinoic acid (0.1 or 10 uM) inhibited anchorage independent growth and decreased the phosphorylation, but not the synthesis of these proteins. Cells that were resistant to treatment with retinoic acid exhibited anchorage independent growth and phosphorylated low molecular weight membrane proteins which supports the possibility that retinoic acid may function by affecting the phosphorylation of specific proteins.

4. Retinoids and glycoconjugate synthesis

Retinoids also affect the synthesis and secretion of glycosaminoglycans, proteoglycans, glycolipids, and glycoproteins, including fibronectin. The effect of retinoids on glycoconjugates varies with the cell strain (reviewed by Lotan, 1980). Very little is yet understood about how these changes affect cell growth and/or transformation.

5. Retinoids and growth factors

The interaction of retinoids with peptide growth factors has been proposed to be a mechanism for the effect of retinoids on carcinogenesis

(Sporn and Roberts, 1983; Jetten, 1984; Sporn et al., 1986). Support of this mechanism is provided by the fact that retinoic acid (1 - 10 uM) increases the number of EGF receptors of several cell types (Jetten, 1980; Jetten, 1982; Jetten and Goldfarb, 1983). Retinoic acid (0.3 uM) was also reported to enhance mitogenesis by EGF of human fibroblasts (Harper and Savage, 1980). However, vanZoelen et al. (1986), with a treatment concentration of 0.2 uM, did not observe this in human fibroblasts, but did report this effect on NRK and 3T3 fibroblasts.

Anchorage independent growth induced by protein growth factors is also affected by retinoic acid. For example, Todaro et al. (1978) reported that retinoids reduced th anchorage independent growth of NRK cells induced to grow by a sarcoma growth factor preparation from conditioned medium of murine sarcoma virus-transformed cells. Also, Roberts et al. (1985) found that the anchorage independent growth of myc-transfected Fischer rat 3T3 fibroblasts that was induced by PDGF with TGF-β was inhibited by retinoic acid (1.0 nM). This growth induced by EGF was also reduced by retinoic acid (100 nM), but was not as sensitive to the effect as the growth induced by PDGF with TGF-β. In contrast to these reports, Jetten and Goldfarb (1983) and van Zoelen et al. (1986) have reported that retinoic acid (0.2 - 1.0 uM) increases the anchorage independent growth of NRK cells that was induced by specific peptide growth factors. The above research indicates that retinoic acid can affect anchorage independent growth induced by specific peptide growth factors of various cells and that the effect may be dependent on the type of cell. However, the concentrations employed by these investigators, with the exception of Roberts et al. (1985), were pharmacological rather than physiological.

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Chapter 2

Platelet-Derived Growth Factor or Basic Fibroblast Growth Factor Induce Anchorage Independent Growth of Human Fibroblasts

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Abstract

Anchorage independent growth, i.e., growth in semi-solid medium is considered a marker of cellular transformation of fibroblast cells. Diploid human fibroblasts ordinarily do not exhibit such growth, but can grow transiently when medium contains high concentrations of fetal bovine serum. This suggests that some growth factor(s) in serum is responsible for anchorage independent growth. Much work has been done to characterize the peptide growth factor requirements of various rodent fibroblast cells for anchorage independent growth, however, the requirements of human fibroblasts are not unknown. To determine the peptide growth factor requirements of human fibroblasts for anchorage independent growth, we used medium containing serum that had had its peptide growth factors inactivated. We found that either platelet-derived growth factor (PDGF) or the basic form of fibroblast growth factor (bFGF) induced anchorage independent growth. Epidermal growth factor (EGF) did not enhance the growth induced by PDGF, or did so only slightly. Transforming growth factor beta (TGF-β) decreased the growth induced by PDGF. EGF combined with TGF-β induced colony formation in semi-solid medium at concentrations at which neither growth factor by itself was effective, but the combination was much less effective in stimulating anchorage independent growth than PDGF or bFGF. This work showed that PDGF, or bFGF, or EGF combined with TGF-β can stimulate anchorage independent growth of non-transformed human fibroblasts. The results support the idea that cellular transformation may reduce or eliminate the need for exogenous PDGF or bFGF.

Introduction

The transition of a normal fibroblast in culture to a tumor forming cell has been proposed to involve several stable changes (Land et al., 1983). One of these changes is the ability to exhibit anchorage independent growth, a phenotype that has been found to correlate highly with tumorigenicity in mouse fibroblasts (Shin et al., 1975). Several groups of investigators have shown that carcinogen treatment induces human fibroblasts to grow in anchorage independent conditions (Freedman and Shin, 1977; Milo and DiPaolo, 1978; Sutherland et al., 1980; Silinkas et al., 1981; Maher et al., 1982). Milo and DiPaolo (1978) also reported that the carcinogen transformed cells were capable of producing nodular growths in athymic mice. This suggests that human fibroblasts that acquire the property of anchorage independent growth may have acquired one of the stable properties involved in malignant transformation.

Anchorage independence, which is characteristic of these transformed fibroblasts, can also be found transiently in non-transformed fibroblasts if the medium contains high concentrations of serum. (Peehl and Stanbridge, 1981; McCormick et al. 1985). This indicates that serum contains component(s) which can induce transient anchorage independent growth. Extensive work on the induction of anchorage independent growth of rodent fibroblasts indicates that certain protein growth factors can promote this growth. Roberts et al. (1982) reported that transforming growth factor beta (TGF-β) was able to stimulate the anchorage independent growth of normal rat kidney (NRK) cells in a serum-containing medium. Subsequently, others also reported that

addition of various protein growth factors to medium containing serum increased the frequency of anchorage independent colonies formed by rodent cells. For example, Anzano et al. (1986) showed that platelet-derived growth factor(PDGF) induced anchorage independent growth of primary rat embryo fibroblasts and NIH 3T3 mouse cells. Epidermal growth factor (EGF) combined with TGF-β also induced growth of anchorage independent colonies, but the combination of these two growth factors was not as effective as PDGF. Stern et al. (1986) reported that Fisher rat 3T3 cells transfected with the c-myc gene exhibited anchorage independent growth when EGF was added to the medium. PDGF or TGF-β were less effective in their assay. These studies support the idea that protein growth factors are involved in stimulating anchorage independent growth. However, since serum provides an unknown amount of various protein growth factors, including PDGF and TGF-β, it is difficult to clearly determine which factors are necessary for anchorage independent growth.

In order to determine the role of specific protein growth factors in the induction of anchorage independent growth, some investigators have used medium lacking serum or medium containing serum which has had its peptide growth factors inactivated by cleavage and reduction of the disulfide bonds with dithiothreitol and iodoacetamide. For example, McClure (1983) using serum-free medium, showed that PDGF or fibroblast growth factor (FGF) stimulated anchorage independent growth of SV40 transformed BALB/c-3T3 mouse cells. Under similar conditions, NRK cells were also induced to grow in anchorage independent conditions

by PDGF (Rizzino et al., 1986) or by the basic form of FGF (bFGF) (Rizzino and Ruff, 1986). In contrast, when van Zoelen et al. (1986) used a system that employs growth factor-inactivated serum, they found that PDGF by itself did not stimulate anchorage independent growth of NRK cells. However, when combined with EGF and TGF-β, PDGF stimulated such growth. The combination of EGF and TGF-β without PDGF was capable of stimulating anchorage independent growth of these cells, but not as strongly. Later, van Zoelen et al. (1988) reported that PDGF alone at high concentrations was capable of stimulating anchorage independent growth.

From these investigations, it appears that the role of growth factors in inducing anchorage independent growth is complex. The present studies were undertaken to investigate the growth factor requirements for anchorage independent growth of diploid human fibroblasts. The results indicated that PDGF or bFGF induce anchorage independent growth, but neither EGF nor TGF-β alone have an effect. EGF with TGF-β also stimulated anchorage independent growth, but was much less effective than PDGF or bFGF.

MATERIALS AND METHODS

Cells and culture medium

Diploid human fibroblast cultures were derived from foreskin material of normal newborn males (McCormick and Maher, 1981). Cells were routinely cultured in McM medium, a modification of Ham's MCDB 110 medium (Bettger et al., 1981), as described by Ryan et al. (1987). Unless otherwise designated, this medium was supplemented with 10% fetal bovine serum (FBS) from Gibco Laboratories, (Grand Island, N.Y.), penicillin (100 U/ml) and streptomycin (100 µg/ml). The cultures were maintained in a humidified incubator at 37°C and an atmosphere of 5% CO_2 and 95% air.

For the anchorage independence assay utilizing growth-factor inactivated serum, the peptide growth factors were inactivated by treatment of FBS with dithiothreitol and iodoacetamide (van Zoelen et al., 1985). The McM medium containing growth-factor inactivated serum (9% v/v) was also supplemented with insulin (1 µg/ml) and ferrous sulfate (1.39 µg/ml). The latter two were prepared and added as described by Ryan et al. (1987). After several experiments had been completed, it was determined that insulin was not necessary in the medium for anchorage independent growth (data not shown). Since inactivation and subsequent dialysis of the FBS resulted in loss of protein and lipids, a lipid supplement and bovine serum albumin were added to the medium. The lipid supplement contained cholesterol (3 mg/ml), sphingomyelin (1 mg/ml), soybean lecithin (6 mg/ml), and tocopherol (60 µg/ml) and tocopheryl acetate (0.2 mg/ml). These lipid compounds were dissolved or suspended in ethanol, and diluted into a 5% stock solution of bovine serum albumin and the mixture was diluted into the medium to give a 0.5% final concentration of bovine serum albumin (Ryan et al., 1987).

Chemicals

EGF from mouse submaxillary gland and PDGF from porcine platelets were purchased from Bethesda Research Laboratory (Gaithersburg, MD). During the time of these experiments the supply of porcine PDGF became unavailable; therefore, some of the experiments were conducted with human PDGF. The form used in each experiment is indicated in the Table or Figure legends. PDGF purified from human platelets was obtained from PDGF, Inc. (Boston, MA). Purified bFGF from bovine pituitary was bought from Collaborative Research Inc. (Bedford, MA). TGF-β was kindly supplied by Dr. Richard Assoian of Columbia University.

Anchorage independence assay

Exponentially growing cultures between population doublings 15 and 20 were used for the anchorage independence assay. The medium was replaced with fresh medium 24 hours prior to the assay to ensure exponential growth. Anchorage independent growth was measured in the presence of growth factor inactivated serum (van Zoelen, et al., 1985), dialyzed FBS, or FBS. Cells were suspended in medium containing 0.3% SeaPlaque or SeaKem agarose (FMC Inc., Rockland, ME) (2000 cells in 0.6 ml). This medium was layered on a hardened base layer of medium containing 2.0% agarose in a 35 mm tissue culture dish. The agarose was allowed to harden for one hour at room temperature before incubation. Medium (1.4 ml) containing the designated growth factor(s) was added 6-12 hr later. The final concentration of the growth factor(s) was based on the total volume of the system (3.5 ml). The dishes were incubated for 2-3 weeks at 37°C in an atmosphere of 3% CO_2/97% air. After one week, the medium above the top agar layer was removed and

replaced with fresh medium containing the designated growth factor(s). One week later, colonies >60 μm in diameter were counted using an inverted microscope with a calibrated grid in the eyepiece.

RESULTS

Effect of protein growth factors in the presence and absence of serum

The effect of EGF, PDGF or TGF-β on anchorage-independent growth was first investigated in McM medium supplemented with FBS. In the presence of 5% FBS, either EGF or PDGF increased the frequency of anchorage-independent growth of diploid human fibroblasts (Table 1). TGF-β decreased the number of anchorage-independent colonies in the presense of 5% FBS.

Since decreasing the serum concentration decreased the number of anchorageindependent colonies, the effect of protein growth factors on anchorage-independent growth in the absence of serum was investigated. A serum-free medium which supports anchorage dependent growth of human fibroblasts (Ryan et al., 1987) was used. Under these conditions, neither EGF nor PDGF stimulated anchorageindependent growth (data not shown). Since Roberts et al. (1983) had reported that both EGF and TGF-β were required for anchorage-independent growth of NRK cells, we also tested the interactive effects of different combinations of PDGF, EGF or TGF-β . Again, no anchorage-independent growth was not observed (data not shown).

Effect of protein growth factors in the presence of growth factor-inactivated serum

Since serum was apparently providing an unknown component necessary for anchorage-independent growth and we wanted to study the effects of exogenous protein growth factors in the absence of these factors in serum, we used FBS which had had the growth factors inactivated.

TABLE 1. Anchorage independent growth response of diploid human fibroblasts to EGF (mouse), PDGF (human) or TGF-β (human) in the presence of serum[1]

Percent serum	Growth factor tested[2]	Number of colonies		
		Exp 1	Exp 2	Exp 3
5.0	None	154±21	204±6	178±16
5.0	EGF (30)	196±10	383±15	
5.0	PDGF (0.5)	303±10	533±5	
5.0	TGF-β (0.1)	88±17	183±10	67±10
1.0	None	0	0.3±5	0
1.0	EGF (30)	8±2	36±5	
1.0	PDGF (0.5)	99±12	242±13	
1.0	TGF-β (0.1)	0	0	0

[1]The results of each treatment effect are expressed as the average and standard error of 3-4 dishes each containing 2,000 cells.

[2] The numbers in parentheses are the concentration of the growth factor in ng/ml.

Growth factor-inactivation was accomplished by treatment of FBS with dithiothreitol and iodoacetamide as described by van Zoelen et al. (1985). The latter two chemicals were removed by extensive dialysis against phosphate-buffered saline (PBS). The effect of various exogenous growth factors on anchorage-independent growth in the presence of this growth factor-inactivated serum was compared to the effect in medium supplemented with non-treated FBS (10% v/v) that had also been dialyzed against PBS. No anchorage-independent growth, was observed in dishes with no added growth factor. As shown in Figure 1, either PDGF or bFGF stimulated anchorage-independent growth with a dose dependent response. Neither EGF (30 ng/ml) nor TGF-β (1.0 ng/ml) was effective in stimulating anchorage-independent growth. We also examined the effects of various combinations of growth factors (Table 2). EGF with TGF-β induced anchorage-independent growth at concentrations at which neither growth factor alone was effective. EGF enhanced the growth induced by PDGF in two of the three experiments. TGF-β decreased the anchorageindependent growth induced by PDGF or by EGF with PDGF.

TABLE 2. Interactive effects of growth factors on anchorage independent growth of human fibroblasts in medium containing growth factor inactivated serum[1]

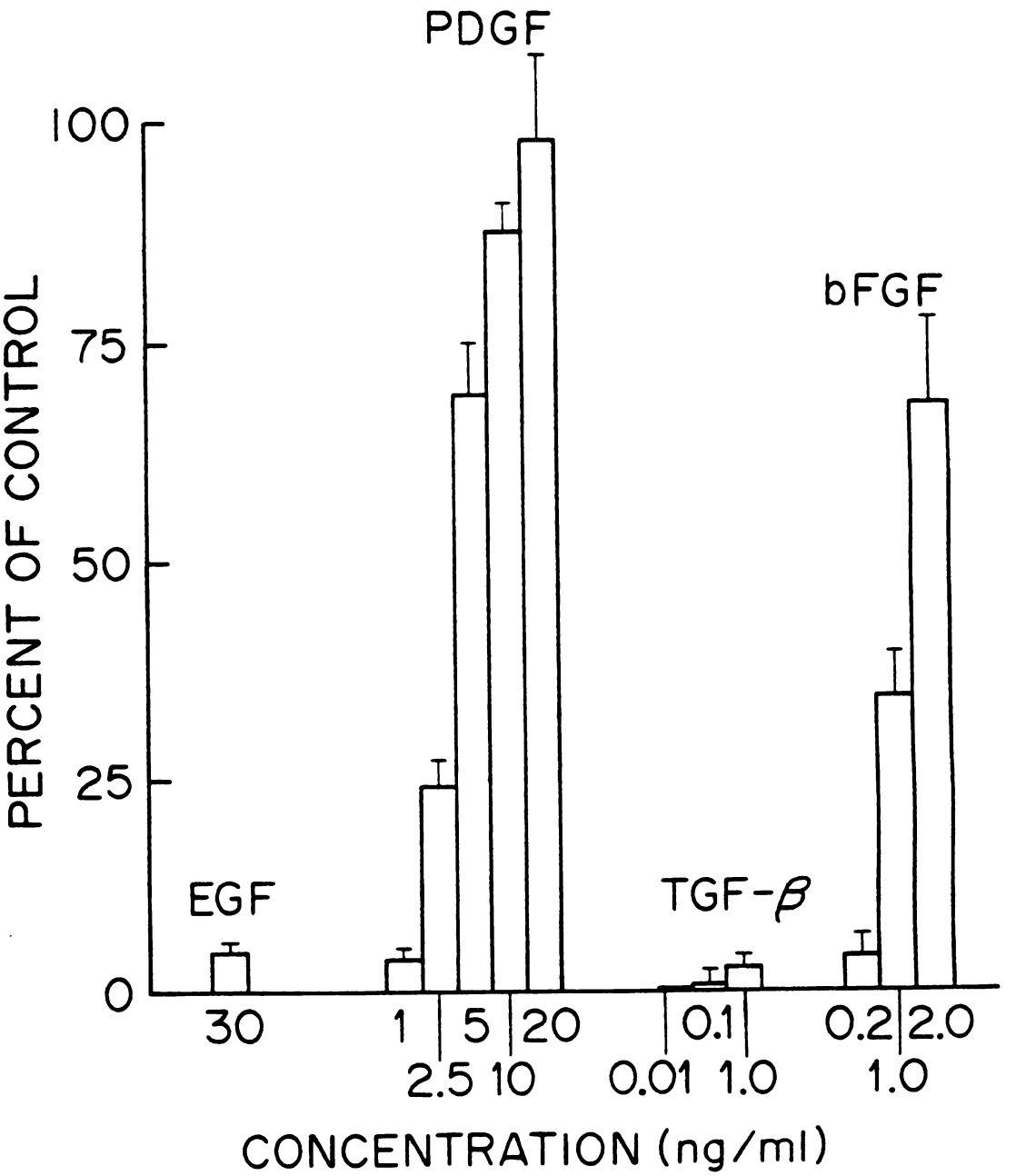
Growth factor tested[2]	Number of Colonies		
	Exp 1	Exp 2	Exp 3
Serum - 10%[3]	51 ± 10	60 ± 3	46 ± 5
None	0	0	0.3 ± 0.3
EGF (30) + TGF-β (0.1)	11 ± 3	--	4 ± 2
PDGF (10)	61 ± 14	52 ± 2	30 ± 1
PDGF (10) + EGF (30)	97 ± 12	47 ± 9	54 ± 6
PDGF (10) + TGF-β (0.1)	40 ± 4	--	15 ± 3
PDGF (10) + TGF-β (1.0)	--	--	10 ± 2
PDGF (10) + EGF (30) + TGF-β (0.1)	48 ± 4	--	15 ± 5

[1] The results of each treatment effect are expressed as the average and standard error of 3-4 dishes each containing 2,000 cells.

[2]The numbers in parentheses are the concentration of the growth factor in ng/ml. The source of PDGF is porcine.

[3]The results obtained in medium containing dialyzed 10% FBS are included as a control.

Fig. 1. Anchorage independent growth induced by specific growth factors in the presence of growth factor inactivated serum. The results are expressed as percentage of the anchorage independent growth observed using McM medium supplemented with 10% dialyzed FBS (frequencies ranging from 2-3%). The response to EGF or bFGF represents the average of two experiments, with 3 plates per experiment. The response to TGF-β at a concentration of 0.1 ng/ml was tested in three experiments; that at 1.0 ng/ml was tested in one experiment. The dose response of PDGF (porcine) was taken from one experiment. The data are representative of the response seen for the various concentrations of PDGF measured in a series of experiments.


PDGF
bFGF
EGF
TGF-β
100
75
50
25
0
PERCENT OF CONTROL
30
1
5
20
0.1
0.2
2.0
2.5
10
0.01
1.0
1.0
CONCENTRATION (ng/ml)

DISCUSSION

The results in Table 1 and Figure 1 show that PDGF or bFGF stimulate anchorage independent growth of human fibroblasts in medium supplemented with FBS or with growth factor inactivated FBS. Others have reported similar results with rodent cells. Anzano et al. (1986) reported that in medium containing serum, PDGF is the strongest stimulator of anchorage independent growth of primary rat embryo fibroblasts. Also, van Zoelen et al. (1988) reported that PDGF induced anchorage independent growth of NRK cells in medium containing growth factor inactivated serum.

In contrast to our results in medium lacking serum, Rizzino et al. (1986) reported induction of anchorage independent growth of NRK cells by PDGF. Since their medium contained high density lipoprotein (HDL) as the lipid source, we tested human high density lipoproteins (HDL, 75 ug/ml) or low density lipoproteins (LDL, 2.5 and 25 ug/ml) (Meloy Laboratories, Springfield, VA) as the lipid source in our serum-free medium (data not shown). We found that HDL did support growth induced by PDGF, but at a much lower level than either HDL or our regular lipid source did in the presence of growth factor-inactivated serum. LDL was less effective. Since we were unable to detect stimulation of anchorage independent growth of diploid human fibroblasts by PDGF in serum-free medium, we conclude that growth factor-inactivated serum was supplying one or more components, other than peptide growth factors, which are necessary to support anchorage independent growth.

Also, unlike our results, Rizzino et al. (1986) reported that PDGF did not induce anchorage independent growth of NRK cells in medium

containing serum. As these investigators suggested, the TGF-β content of the serum may have decreased the ability of PDGF to induce anchorage independent growth of these cells. It is generally recognized that different sources of FBS have varying concentrations of growth factors. The FBS used in our laboratory is screened for its ability to support cloning and bulk growth of human fibroblasts. Therefore, it is likely that we used serum with a minimum concentration of inhibitory substances.

Our results support the idea that acquisition of the ability to synthesize PDGF or bFGF represent a step in cellular transformation. The concept that growth factors are involved in malignant transformation has received support from many studies. For example, many tumor-derived cell lines no longer require specific growth factors in the medium (Kaplan et al., 1982). Autocrine stimulation of growth by "turning on" endogenous synthesis of a growth factor is one possible mechanism for a cell to eliminate or substantially reduce the requirement for a specific exogenous growth factor. Since the transforming oncogene v-sis has homology to the gene for the B chain of PDGF (Doolittle et al., 1983), it is possible that activation of the c-sis protooncogene is a step in the transformation of those cell types which respond to PDGF as a growth factor. This hypothesis is consistent with the reports that, unlike normal human fibroblasts, human fibrosarcoma-derived HT1080 cells synthesize m-RNA for the B chain of PDGF (Eva et al., 1982) and secrete PDGF-like proteins (Pantazis et al., 1985). It is also consistent with the finding that, unlike normal fibroblasts, HT1080 cells are capable of anchorage independent growth in the absence of exogenous PDGF (H. Palmer, unpublished data) and can be propagated

continuously in serum-free medium without the addition of protein growth factors, such as PDGF (McCormick et al., 1987). Recent studies have shown that diploid human fibroblasts transfected with the v-sis oncogene are capable of growing continuously in the absence of serum, synthesize m-RNA for PDGF, form foci and grow to high saturation density, and exhibit anchorage independent growth (Fry et al., 1986 and Stevens et al., 1988). Further studies with transformed human fibroblasts that exhibit anchorage independent growth are being conducted to determine their ability to synthesize PDGF or bFGF.

ACKNOWLEDGEMENTS

We would like to thank Dr. Maurice Bennink for reading the manuscript, Carol Patterson for the typing and Dr. Dennis Gilliland for his advice on data analysis. This research was supported in part by Department of Energy Grant DE-F602-87ER-60524, by DHHS grants CA21289 and CA32490 from the NCI, and a gift from the Department of Michigan Ladies' Auxiliary to the Veterans of Foreign Wars.

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Chapter 3

The Effect of Retinoids on Growth Factor-Induced Anchorage Independent Growth of Human Fibroblasts

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SUMMARY

We have studied the effects of all-trans retinol, all-trans retinoic acid and anhydroretinol, a non-biologically active retinoid, on anchorage independent growth of human fibroblasts induced by purified growth factors. The anchorage independence assay was conducted in medium supplemented with serum that had had its peptide growth factors inactivated by treatment with dithiothreitol and iodoacetamide. The ability of normal human fibroblasts to grow in semisolid medium when induced by platelet-derived growth factor (PDGF) was reduced by physiologic concentrations of either all-trans retinol (0.5 uM) or all-trans retinoic acid (1.0 nM) but not by anhydroretinol (0.5 uM). The frequency of anchorage independent growth induced by basic fibroblast growth factor (bFGF) was also reduced by all-trans retinol. All-trans retinol also reduced the frequency of anchorage iondependent growth of the human fibrosarcoma-derived cell line, HT1080, which grew in semisolid medium without added growth factors. These data show that retinoids reduce the frequency of anchorage independent growth of human fibroblasts induced by either PDGF or bFGF. Since PDGF and bFGF bind to independent cell membrane receptors and are known to stimulate different pathways leading to DNA synthesis, the data suggest that physiologically active retinoids have an effect on a step which is common to both signal pathways.

INTRODUCTION

The ability of retinoids, a class of compounds which includes vitamin A, to prevent the development of malignant tumors or retard their rate of growth has been reported in epidemiological studies (Bjelke, 1974; Ziegler et al., 1981; Kolonel, 1985), experimental studies using animal models (Bollag, 1971; Moon et al., 1983; Longnecker et al., 1983; Zile et al., 1986), and clinical studies (Peck, 1983; Peck, 1987; Lippman and Meyskens, 1987; Kraemer et al., 1988). The majority of these experimental investigations used pharmacologic amounts of retinoids. Several groups of investigators have examined the effects of retinoids on mammalian cells in culture in an attempt to understand how they might affect tumor formation. The results of studies with established rodent fibroblast cell lines show that retinoids can inhibit cell proliferation (Lotan and Nicolson, 1977; Dion et al., 1977; Lotan, 1980), increase cell-to-substratum adhesiveness, and reduce the number of cells found at confluence (Jetten et al., 1979; Kamei, 1983). In studies with human fibroblasts, Lacroix et al. (1980) reported that retinoids caused inhibition of the growth of three of the four cell lines tested. Harper and Savage (1980), using one of the same retinoids at approximately the same concentrations, did not observe this effect on the growth of human fibroblasts.

Others have examined the ability of retinoids to prevent the expression of a transformed phenotype in rodent fibroblast cell lines transformed in culture by viruses or carcinogens. For example, Merriman and Bertram (1979) and Bertram (1980) showed that retinyl acetate and several synthetic retinoids could prevent the formation of foci by

C3H/10T1/2 cells that had been transformed by a chemical carcinogen. Similarly, Dion et al. (1978) and Mukherjee et al. (1983) showed that retinoic acid could prevent the formation of colonies in semisolid medium by transformed cells (anchorage independent growth). Freedman and Shin (1974) and Shin et al. (1975) showed that anchorage independent growth of rodent cells transformed in culture is highly correlated with development of nodular tumors and Milo et al. (1981) showed this same correlation for carcinogen-treated diploid human fibroblasts.

Since protein growth factors have been shown to induce anchorage independent growth of rodent fibroblasts (McClure, 1983; Rizzino et al., 1986; Rizzino and Ruff, 1986) and of human fibroblasts (Palmer et al., 1988), it was of interest to determine the effect of retinoids on this induction by growth factors. Therefore, in the present study we investigated the effect of several retinoids on the frequency of anchorage independent growth of diploid human fibroblasts induced by platelet-derived growth factor (PDGF) or basic fibroblasts growth factor (bFGF). We have also examined the ability of human fibrosarcoma-derived HT1080 cells to form colonies in semisolid medium in the absence of exogenous protein growth factors and determined the effect of all-trans retinol on this anchorage independent growth. The results showed that retinoids can decrease the frequency of growth factor-induced anchorage independence in normal human fibroblasts and can also decrease the frequency of anchorage independent growth of HT1080 cells.

MATERIALS AND METHODS

Reagents

Platelet-derived growth factor purified from human fibroblasts was purchased from PDGF, Inc. (Boston, MA). A highly-purified basic form of fibroblast growth factor derived from bovine pituitary was purchased from Collaborative Research (Bedford, MA). Epidermal growth factor (EGF) was purchased from Bethesda Research Laboratories (Gaithersburg, MD). All-trans retinol and all-trans retinoic acid were obtained from Sigma Chemical Co. (St. Louis, MO). The retinoids were stored at -20°C under nitrogen. Work with the retinoids was conducted under "gold" fluorescent lights. Stock solutions were freshly prepared every two weeks.

Synthesis of anhydroretinol

Anhydroretinol was synthesized from all-trans retinol according to the procedure described by Shantz et al. (1943). The synthetic reaction product was extracted 4 times with 5 ml hexane. The extract was evaporated under nitrogen to a minimal volume and purified by elution from an alumina column. The alumina column was prepared by combining 1 ml of water with 12.5 g of activated aluminum hydroxide (Brockmann 1, 150 mesh; Aldrich Chemical Co., Milwaukee, WI), making a slurry of the alumina with hexani, pouring the mixture into a 25 ml buret, and rinsing the column with hexane. The ultraviolet absorption spectrum of the eluted fractions (5 ml) was scanned. Fractions with the characteristic absorption maxima at 351, 371 and 392 mn were pooled, the hexane was evaporated under nitrogen, and the sample residue was dissolved in ethanol to give the

appropriate concentration. The purity of the preparation, as determined from the ratio of E(392)/E(371) and E(351/E(371) (Budowski and Bond, 1957), was 98%.

Cells and culture conditions

Diploid human fibroblast cultures were derived from foreskin material of normal newborn males (McCormick and Maher, 1981). HT1080, a human fibrosarcoma-derived cell line, was obtained from the American Type Culture Collection (Rockville, MD). The cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

Inactivation of growth factors in serum

Peptide growth factors in fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, NY) were inactivated by reduction and acetylation of disulfide bonds by treatment with dithiothreitol (Boehringer-Mannheim, Indianapolis, IN) and iodoacetamide (Sigma Chemical Co.) as described by van Zoelen et al. (1985). Extensive dialysis of the treated serum against phosphate buffered saline (PBS) was conducted to remove the chemicals from the preparation. Untreated FBS dialyzed against PBS was used as a control.

Media

McM medium, the modification of Ham's MCDB 110 medium (Bettger et al., 1981) described by Ryan et al. (1987), was used. For routine culturing, this medium was supplemented with 10% FBS and antibiotics (penicillin, 100 U/ml and streptomycin, 100 ug/ml). Unless otherwise

indicated, the anchorage independence assays were conducted with McM medium supplemented with 9% growth factor-inactivated FBS and antibiotics. Since the inactivation procedure caused precipitation of protein and loss of lipids, bovine serum albumin (BSA) (Fraction V, A-4503, Sigma Chemical Co.) and a lipid supplement were added to the medium. The lipid supplement contained cholesterol (3 mg/ml), sphingomyelin (1 mg/ml), soybean lecithin (6 mg/ml), and tocopherol (60 ug/ml) and tocopheryl acetate (0.2 mg/ml). As described by Ryan et al. (1987) these lipid compounds were dissolved or suspended in ethanol and diluted into a 5% stock solution of BSA. The mixture was diluted into the medium to give a final concentration of BSA of 0.5%. The medium was also supplemented with insulin (1 ug/ml) and ferrous sulfate (1.39 ug/ml). The latter two compounds were prepared and added as described by Ryan et al. (1987). McM medium supplemented as described here for the anchorage independence assay will be referred to as GFIS-medium for convenience.

Assay of cytotoxicity from loss of colony-forming ability

The cytotoxic effect of the retinoids was determined from their effect on the cloning efficiency of the cells. Cultures of the cells were dislodged with trypsin and diluted into McM medium supplemented with 10% FBS and plated into 60-mm-diameter culture dishes at 50 cells per dish. The test compounds dissolved in ethanol were added to the medium in the dishes by micropipette 6-12 hr after plating the cells and twice a week for the duration of the experiment. The volume of ethanol added to the medium was 0.25% of the total volume. After one week, the medium was exchanged for fresh medium containing the test retinoids. The colonies

were stained with crystal violet after two weeks and the number of colonies in the treated dishes was compared to that in the control dishes which received solvent only.

Assay of cytotoxicity from the rate of cell growth

Cells (1×10^3) were plated in 60-mm-diameter dishes. The test compound dissolved in ethanol or the solvent control was added to the dishes twice a week. The medium was replaced with fresh medium after one wk of incubation. Over the course of 11 days, the number of cells in duplicate dishes from each treatment group was determined with the aid of an electronic particle counter (Coulter Corp., Hialeah, FL).

Anchorage independence assay

For this assay, cells in early passage (less than 20 population doublings) were maintained in exponential growth in McM medium supplemented with 10% FBS. The medium was renewed 24 h prior to the assay to ensure exponential growth. To assay anchorage independent growth, the cells were suspended in GFIS-medium or other designated test media containing 0.33% SeaPlaque or SeaKem agarose (FMC Corp., Rockland, ME) (2000 cells in 0.6 ml). The top agarose medium (0.6 ml) was layered on a hardened base layer of the same test medium containing 2.0% agarose in 35-mm-diameter dishes. The top agarose layer was allowed to harden for 1 h at room temperature before incubating the dishes at 37°C. Liquid test medium (1.4 ml) of the same composition as used in the agarose layers, and also containing the designated growth factor(s) and/or retinoids, was added 6-12 h later. The concentration of the growth factor, retinoid, and

ethanol, solvent for the retinoids, in the 1.4 ml of liquid medium was adjusted to give the desired final concentration in the total volume of the system (3.5 ml). Retinoids were added twice a week. The dishes were incubated for 2-3 wks at 37°C in an atmosphere of 3% CO_2/97% air. Each week, the liquid medium above the top agarose layer was removed and replaced with fresh medium containing the designated growth factor and/or retinoid based on a total volume of 3.5 ml. After 14-21 days, colonies with a diameter greater than or equal to 60 um were counted using an inverted microscope with a calibrated grid in the eyepiece.

Quantitation of all-trans retinol

The liquid medium from above the agarose layer in 3 dishes that had been treated with retinol and three control dishes was removed and combined with an equal volume of methanol containing butylated hydroxytoluene (25 ug/ml). The mixture was stored at -20°C until extracted for analysis by high performance liquid chromatography (HPLC). The internal standard, 13-cis-N-ethyl retinamide (600 ng), in ethanol was added prior to extraction. No more than 36 h prior to analysis, the samples were extracted 4 times with 6 ml of hexane. The extract was dried under nitrogen and redissolved in 500 ul methanol. The sample (80 ul) was analyzed by reverse phase HPLC essentially as described by Cullum and Zile (1986). Two columns were used in tandem: a 70 mm x 4.6 mm pre-column containing Co-Pell ODS (Whatman, Clifton, NJ) and a 250 mm x 4.6 mm ODS-2 column (Whatman). The HPLC equipment consisted of a pump, a mercury lamp UV detector with a 350-nm interference filter, a reporting integrator used for solvent programming, two miniature inert valves, and an automatic

sampling unit (Cullum and Zile, 1986). The solvents used, i.e., methanol:water (88:12) for 6 min and methanol:chloroform (83:17) for 10 min, allowed the detection of any metabolites formed from the retinol during the 2 wk incubation, as well as quantitation of the amount of retinol. Quantitation of all-trans retinol was conducted using the internal standard normalization procedure described by Cullum and Zile (1986). The area under the peaks was calculated from the measurements of the height and the width at half height.

RESULTS

Cytotoxicity of the retinoids

In order to be able to use the highest concentration of retinoids that was not significantly cytotoxic to the cells, initial studies of their cytotoxic effects were conducted. Figure 1 shows the cytotoxic effect of all-trans retinol on normal human fibroblasts and the human fibrosarcoma-derived cell line HT1080 plated at cloning densities. A treatment concentration of 0.5 uM was chosen for the anchorage independence assay since at that concentration, cell survival was between 90% and 95% of the untreated control. Similar experiments to determine the effect of all-trans retinoic acid and anhydroretinol on the cloning ability of the cells were also conducted. The highest concentration that did not significantly decrease the cloning ability of the cells was 1.0 nM for all-trans retinoic acid and 1.0 uM for anhydroretinol (data not shown). Anhydroretinol is a biologically inactive retinoid lacking a functional group at the end of the side chain of the molecule (Shantz et al., 1943). The cytotoxicity of 0.5 uM all-trans retinol was also assayed by measuring its effect on the rate of growth of the normal human fibroblast cells and the HT1080 cells plated at higher than cloning densities in medium supplemented with 10% FBS or in GFIS-medium containing EGF as a mitogen. As shown in Figure 2, retinol did not affect the growth of either of these cell lines.

During the anchorage independence assay retinoids were added to the medium twice weekly. Since only the liquid medium over the agarose layers was removed after one week, the concentration of the retinoid was expected to increase in the agarose layers. To determine if the retinoid

Figure 1 - Effect of all-trans retinol on cloning ability. Normal human fibroblasts (•) or HT1080 cells ( ) were plated at cloning densities and treated twice weekly with various concentrations of the retinoid. After 2 weeks, the number of colonies was scored. Results are expressed as percentage of the cloning efficiency of the cells treated with ethanol, the solvent control. The values for the normal fibroblasts represent the mean ± SE of 15 dishes from 3 experiments. The values for the HT1080 cells represent the mean ± SE of 5 dishes from 1 experiment.

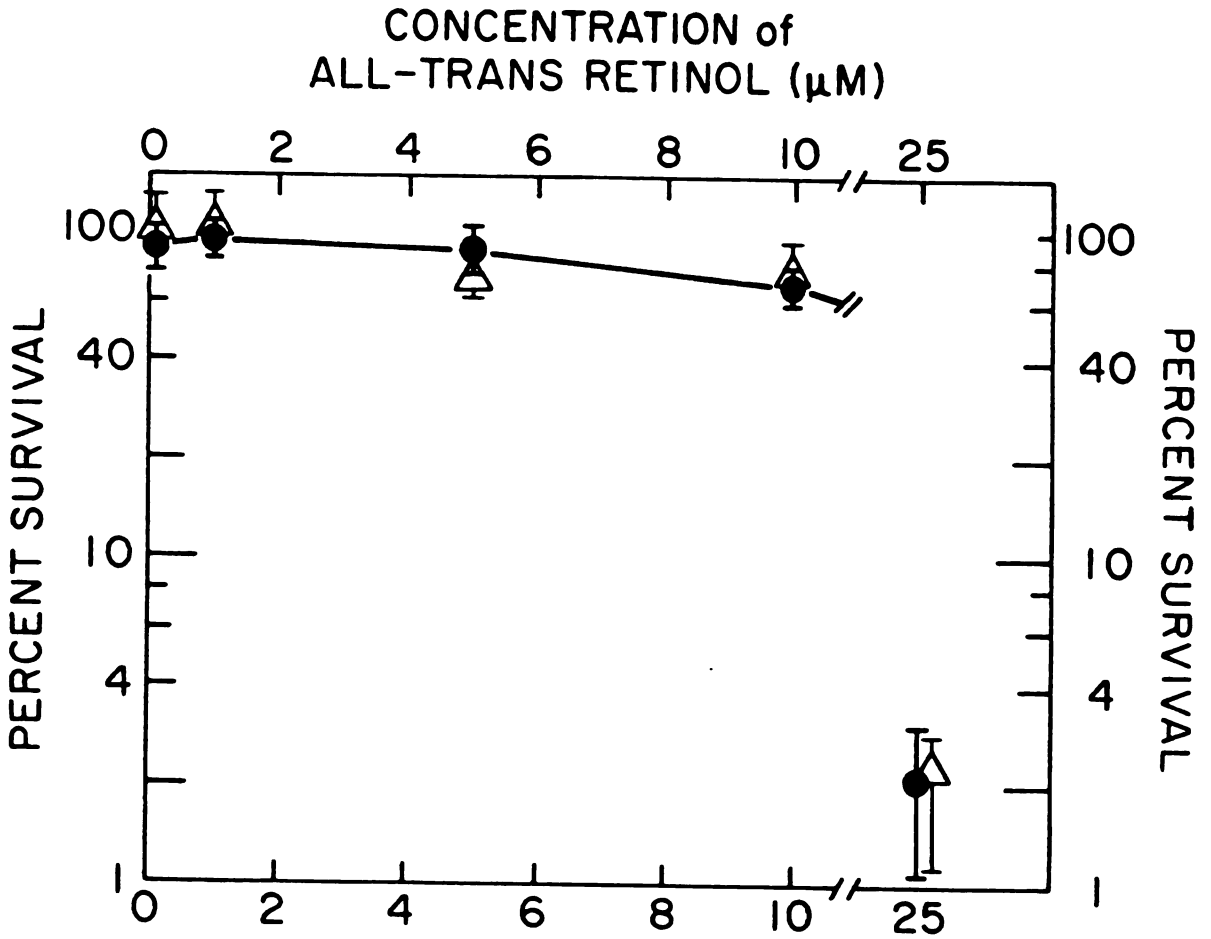
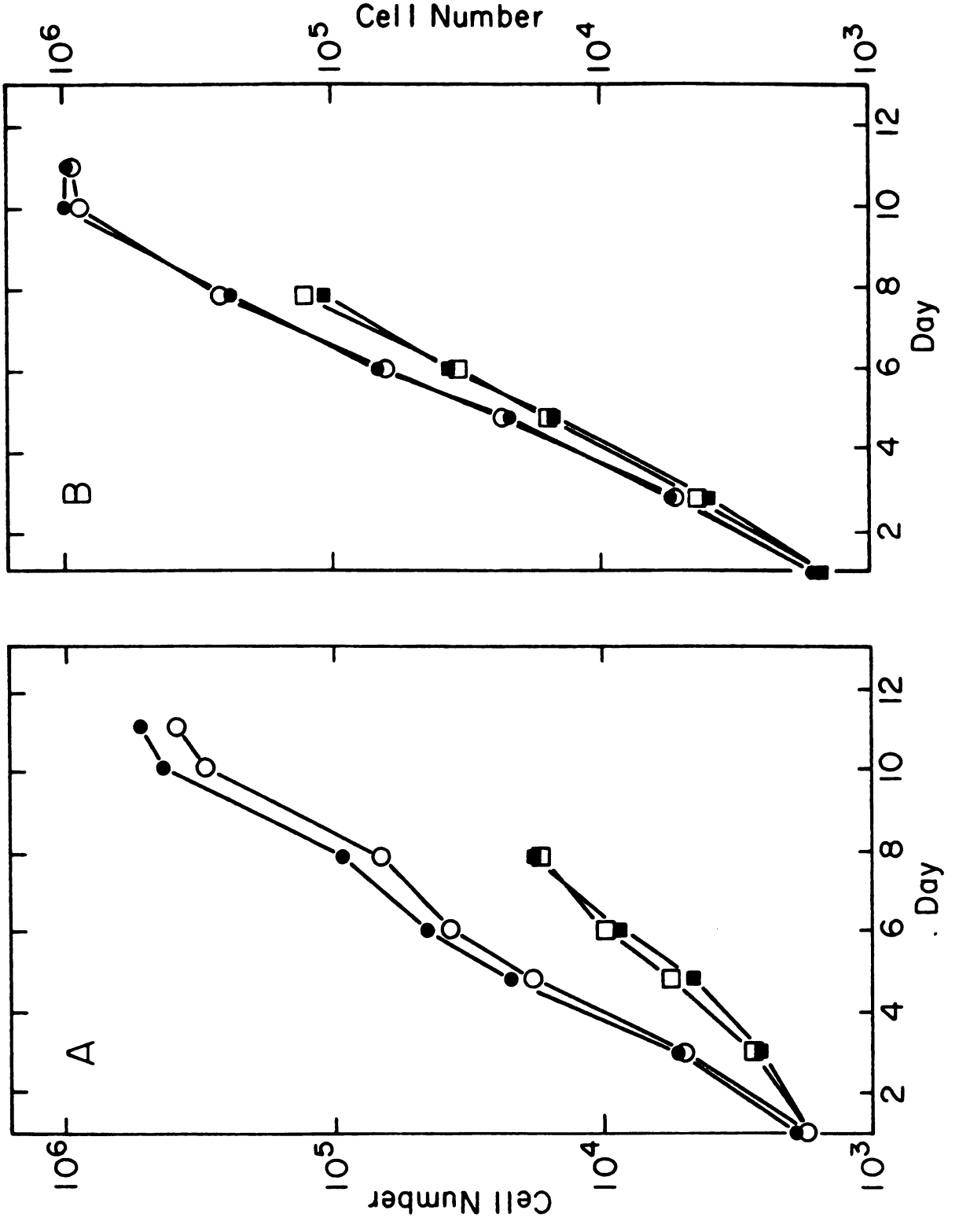

CONCENTRATION of
ALL-TRANS RETINOL (μM)
0
2
4
6
8
10
25
100
40
10
4
1
PERCENT SURVIVAL
PERCENT SURVIVAL

Figure 2 - Effect of all-trans retinol (0.5 uM) on growth of cells. Normal human fibroblasts (A) or HT1080 cells (B) were grown in McM medium supplemented with 10% FBS (,) or GFIS-medium supplemented with EGF (3 ng/ml) (,). The cells were treated twice weekly with ethanol (,) or all-trans retinol (,). The number of cells in duplicate dishes were counted throughout the 11 day experiment.


Cell Number
10^6
10^5
10^4
10^3
B
2
4
6
8
10
12
Day
A
Cell Number
10^6
10^5
10^4
10^3
2
4
6
8
10
12
Day

concentration in the agarose layers had increased to a cytotoxic level during the course of the experiment, the concentration of all-trans retinol in the layer of liquid medium over the agarose layers after 1 and 14 days of incubation was analyzed by HPLC. The results showed that the concentration was 121 ng/ml (0.4 uM) and 464 ng/ml (1.6 uM), respectively. The latter concentration gives a survival of >85% (Figure 1). The profile of the HPLC chromatogram showed no indication of the formation of metabolites of all-trans retinol during this incubation period.

Effect of these retinoids on growth factor-induced anchorage independent growth

As shown in Figure 3, 10% serum supported the anchorage independent growth of normal human fibroblasts, and all-trans retinol decreased the frequency of this growth. The addition of PDGF to this medium greatly enhanced the frequency of anchorage independent growth above that induced by 10% serum alone. The addition of all-trans retinol to the medium containing PDGF and serum resulted in a reduction of the number of anchorage independent colonies. FBS contains a number of growth factors. Therefore, to determine the effect of the retinoids on the frequency of colony formation in agar induced by purified growth factors, we assayed them using GFIS-medium. GFIS-medium was employed, rather than serum-free medium, because anchorage independent growth of normal human fibroblasts can be induced by various protein growth factors in GFIS-medium, but not in serum-free medium (Palmer et al, 1988). Figure 4 shows that GFIS-medium in the absence of PDGF, did not support anchorage independent growth of human fibroblasts. It also shows that all-trans retinol

Figure 3 - Effect of all-trans retinol on serum-induced or PDGF and serum-induced anchorage independent growth. Human fibroblasts (2,000) were assayed for anchorage independence in medium containing 10% FBS in the presence or absence of human PDGF (0.5 ng/ml) and all-trans retinol (Rol, 0.5 uM). The results are the average ± SE of the number of colonies per dish in 8 dishes from 2 experiments. The data were analyzed by one-way analysis of variance and post-hoc treatment comparisons were analyzed by the Scheffe test.

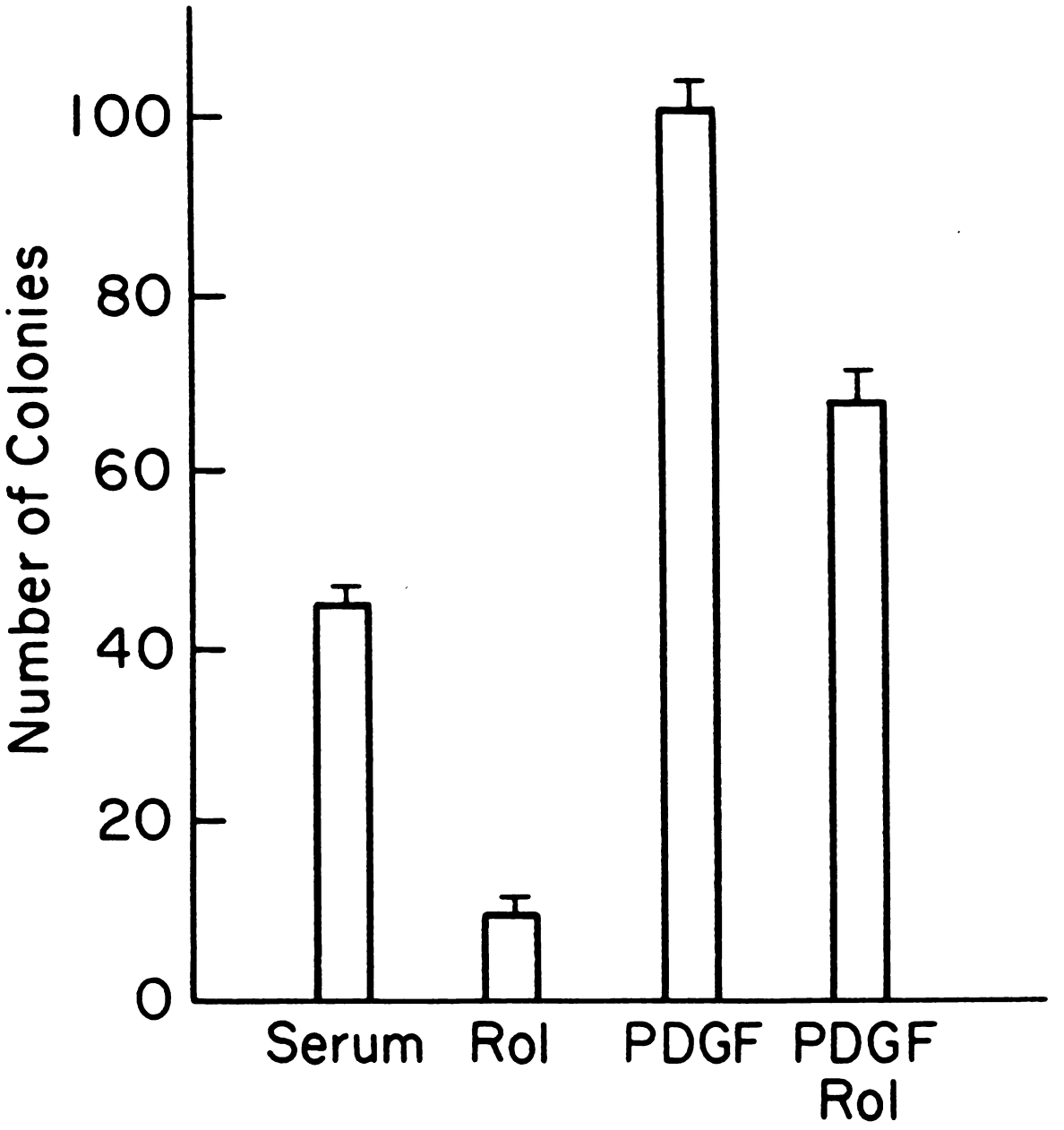

Number of Colonies
100
80
60
40
20
0
Serum
Rol
PDGF
PDGF
Rol

Figure 4 - Effect of all-trans retinol on PDGF-induced anchorage independent growth in GFIS-medium. Human fibroblasts (2,000) were assayed for anchorage independent growth in GFIS-medium in the presence or absence of human PDGF (0.5 ng/ml) and all-trans retinol (0.5 uM). The results are the average ± SE of the number of colonies per dish in 7 dishes from 2 experiments. The data were analyzed by one-way analysis of variance and post-hoc treatment comparisons were analyzed by the Scheffe test.

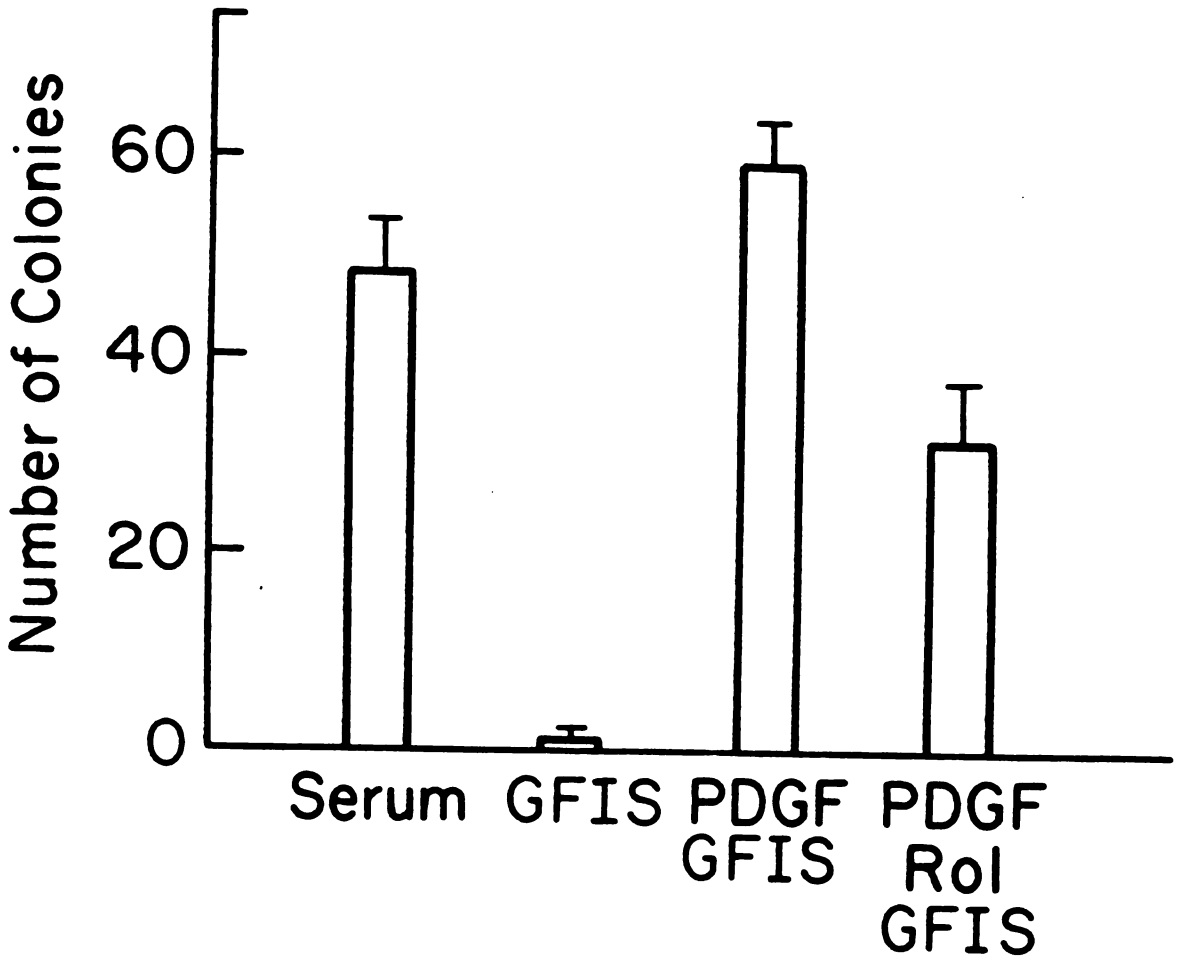

Number of Colonies
60
40
20
0
Serum
GFIS
PDGF GFIS
PDGF Rol GFIS

TABLE 1 - Effect of all-trans retinol on bFGF-induced anchorage independent growth of human fibroblasts in GFIS-medium

Experiment Number	Retinol (0.5μM	Colonies per dish: Amount of bFGF[2]: 0.0	0.2	1.0	2.0
1	-	0	9 ± 1		71 ± 8
	+	0			21 ± 1
2	-	2 ± 1	47 ± 13	173 ± 27	238 ± 13
	+			123 ± 12	146 ± 27

[1]The numbers represent the average ± SE of 3-4 dishes containing 2,000 cells.

[2]The concentration of bFGF in ng/ml.

decreased the frequency of anchorage independent growth induced by PDGF in GFIS-medium. The data in Table 1 indicate that all-trans retinol can also decreased the frequency of colony growth in semisolid medium induced by bFGF. This conclusion is most clearly supported by the data with bFGF at 2.0 ng/ml, but even at 1.0 ng/ml the decrease was greater than the variation indicated by the standard error.

The effect of all-trans retinoic acid (1.0 nM) and anhydroretinol (1.0 uM) on growth factor-induced anchorage independent growth of human fibroblasts was also examined. Anhydroretinol was used as a negative control in the experiment. Figure 5 shows that anhydroretinol did not affect the frequency of anchorage independent colonies of normal human fibroblasts induced by either 10% FBS or PDGF. All-trans retinoic acid, another physiologically active retinoid, was as effective at 1.0 nM as all-trans retinol at 0.5 uM in its ability to decrease the anchorage independent growth of the cells induced by either 10% FBS or PDGF.

Effect of retinol on colony formation in semisolid medium by HT1080 cells

Since HT1080 cells have been shown to synthesize and secrete PDGF-like proteins (Pantazi *et al.*, 1985), and PDGF induces normal human fibroblast cells to form colonies in semisolid medium (Palmer *et al.*, 1988), we tested the ability of HT1080 cells to exhibit anchorage independence in the absence of exogenously-added growth factors. As expected, the HT1080 cells formed colonies in the absence of added growth factors (Table 2). Addition of all-trans retinol decreased the frequency of this colony formation (statistically analyzed by a two-sample *t* test).

Figure 5 - Comparison of the effect of three retinoids on anchorage independent growth of human fibroblasts. Cells (2,000) were assayed for anchorage independent growth induced by 10% FBS (A) or by human PDGF (0.5 ng/ml) in GFIS medium (B) in the absence or presence of either anhydroretinol (1.0 uM), all-trans retinol (0.5 uM), or all-trans retinoic acid (1 nM). The results are expressed as the percent of control (anchorage independent growth induced by 10% FBS (A), or PDGF (B)) and represent the average ± SE of 8 dishes from 2 experiments. The average number of colocies in the 10% serum control was 127±17. The average number of colonies in the PDGF-induced control was 115±10. The decrease in the number of colonies was considered significant based on non-overlapping standard errors.

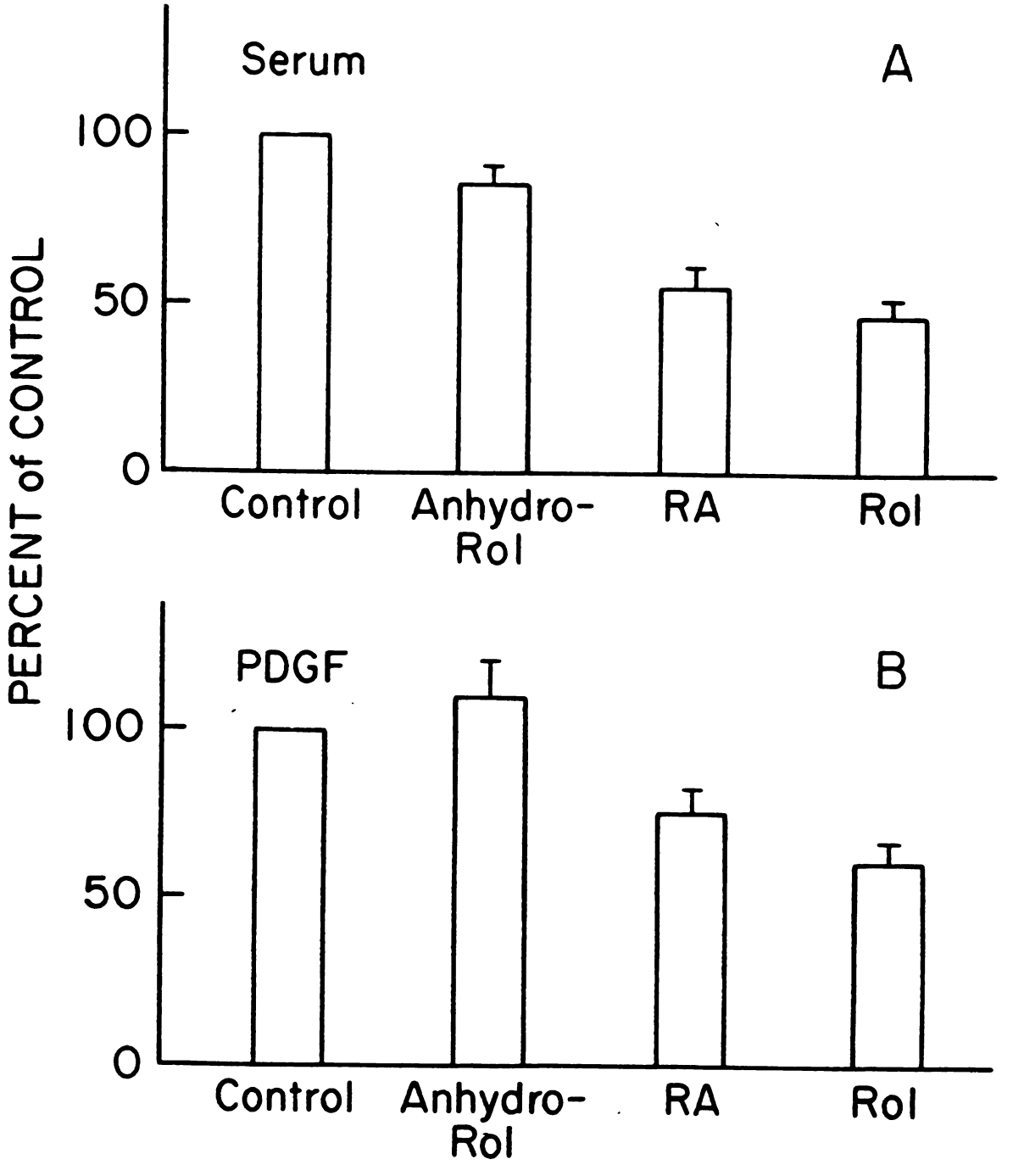

Serum
A
100
50
0
PERCENT of CONTROL
Control
Anhydro-
Rol
RA
Rol
PDGF
B
100
50
0
Control
Anhydro-
Rol
RA
Rol

TABLE 2 - Effect of all-trans retinol on anchorage independent growth of HT1080 cells in the absence of added protein growth factors.

Retinol	Colonies per Dish[1]	
(μM)	Exp. #1	Exp. #2
0.0	53 ± 5	25 ± 3
0.5	30 ± 3	14 ± 4

[1]Numbers represent the average ± SE of 3-4 dishes containing 750 cells.

DISCUSSION

Our results show that non-cytotoxic concentrations of either all-trans retinol or all-trans retinoic acid can reduce the frequency of anchorage independent growth of non-transformed human fibroblasts induced by specific protein growth factors. These data, as well as our results with the HT1080 cells, support the findings of Dion et al. (1978), Lotan et al. (1982), Meyskens et al. (1983), and Mukherjee et al. (1983) that retinoids decrease anchorage independent growth of rodent fibroblast cell lines transformed in culture and of cells derived from human or mouse tumors. They also agree with the results of Roberts et al. (1985) who showed that the frequency of anchorage independent growth of myc-oncogene-transfected Fischer rat 3T3 fibroblasts induced by PDGF and transforming growth factor-β, or by EGF in medium containing 10% calf serum was reduced by retinoic acid. A higher concentration of retinoic acid was necessary to see this reduction of colony formation when induced by EGF than when induced by PDGF and transforming growth factor-β.

In contrast to these reports and our results with human fibroblasts, Jetton and Goldfarb (1983) and van Zoelen et al. (1986) reported that retinoic acid increases the frequency of anchorage independent growth of NRK cells induced by purified protein growth factors. This difference may reflect the fact that retinoic acid is mitogenic for NRK cells, but not for human fibroblasts (van Zoelen et al., 1986). It also increases the number of EGF receptors in NRK cells (Jetton and Goldfarb, 1983).

The majority of reports on the effect of retinoids on cells in culture have tested non-physiologic concentrations. Our results, along with Merriman and Bertram (1979), Bertram (1980), and Roberts et al.

(1985) have shown that physiologic concentrations of retinoids can decrease the frequency of phenotypes that are typically expressed after cellular transformation. However, our results, showing that treatment with either retinol or retinoic acid causes only a 50% reduction in anchorage independent growth of human fibroblasts, indicate that an adequate nutritional intake of vitamin A may not be sufficient to have a significant impact on the development of cancer.

A more comprehensive understanding of both carcinogenesis and the mechanism of the effect of vitamin A can provide a rationale for the usefulness and limitations of retinoids such as vitamin A in the treatment of cancer. Alterations resulting in the constituitive activation of pathways which result in DNA synthesis may be involved in the uncontrolled growth of cells that is basic to cellular transformation and tumorigenesis. The binding of peptide growth factors such as PDGF and bFGF to specific membrane receptors stimulate signal transduction along pathways leading to DNA synthesis. It has been shown in rodent fibroblasts that the signal for DNA synthesis initiated by PDGF binding is mediated by the hydrolysis of phosphatidylinositol bisphosphate and the activation of protein kinase C (Habenicht et al., 1981; Berridge et al., 1984); whereas, the signal transduced after the binding of bFGF is not mediated by this hydrolysis (Chambard et al., 1987; Magnoldo et al., 1986). If this is true in human fibroblasts, our finding that all-trans retinol decreased the frequency of anchorage independent growth induced by either PDGF or bFGF suggests that the effect of retinol is mediated at a common step(s) late in the signal transduction pathway. It is possible that the retinoids may interfere with binding of both of these growth

factors to their specific receptors. However, this does not seem likely since high concentrations of PDGF did not overcome the effect of all-trans retinol (0.5 uM) on the frequency of anchorage independent growth of human fibroblasts by 50% (data not shown).

All-trans retinoic acid at a concentration 1/1000th of all-trans retinol was as effective as all-trans retinol in decreasing the frequency of anchorage independent growth of the human fibroblasts. This is consistent with the fact that all-trans retinol is metabolized to all-trans retinoic acid (Emerick et al., 1967), an active form of vitamin A, but that the reverse reaction does not occur (Dowling and Wald, 1960). Although a peak corresponding to retinoic acid was not seen in the HPLC chromatograph; it may have been present in undetectable, but sufficient amounts for activity.

In summary, the results of the present study provide insight into the mechanisms by which retinoids may prevent or retard tumor development. Further investigations are necessary to determine if the signal transduction pathways for PDGF and bFGF differ in human fibroblasts, and to determine at which step the retinoids interfere in the pathway.

ACKNOWLEDGEMENTS

We would like to thank Carol Patterson for typing this manuscript. This research was supported in part by Department of Energy Grant DE-F602-87ER-60524 and by DHHS grants CA21289 and CA32490 from the NCI.

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Chapter 4

SUMMARY

The results of the work presented in chapters 2 and 3 showed that:

1) anchorage independent growth of human fibroblasts is induced by PDGF or bFGF but not by EGF or TGF-β. EGF with TGF-β, at concentrations at which neither growth factor alone was effective, will induce this growth, but not as well as PDGF or bFGF.

2) all-trans retinol (0.5 uM, a non-cytotoxic concentration) reduces the frequency of anchorage independent growth induced by serum or PDGF or bFGF. All-trans retinoic acid (1.0 nM, non-cytotoxic) is also effective in decreasing this growth induced by serum or PDGF. The biologically inactive retinoid, anhydroretinol, has no effect.

3) the human fibrosarcoma-derived cells, HT1080, which have been reported to secrete PDGF-like proteins, are capable of exhibiting anchorage independent growth in medium supplemented with serum that has had its peptide growth factors inactivated. The frequency of this growth is reduced by all-trans retinol.

Conclusions that come from these results together with the results of other reports are:

1) increases in the amount of vitamin A in the physiologic range are unlikely to have a significant impact on the development of cancer, since only 50% of the number of anchorage indpendent colonies were inhibited by the retinoids tested.

2) the mechanism by which the physiologically active retinoids reduce anchorage independent growth appears to be mediated at a step late in the pathway of signals that lead to DNA synthesis initiated by the binding of a growth factor to its receptor. This conclusion is based on the results that show all-trans retinol decreases anchorage independent growth induced by either PDGF or bFGF and the fact that these two growth factors have several different steps in their mechanisms of transducing the signals leading to DNA synthesis.

The results of this work have raised the following questions for future research:

1) are the pathways leading to DNA synthesis that are initiated by PDGF or bFGF different in human fibroblasts as well as in rodent fibroblasts?

2) are matrix or adhesion proteins such as fibronectin and collagen secreted in response to PDGF or bFGF and are they involved in supporting the ability of human fibroblasts to grow in semisolid medium?

3) what step does vitamin A affect in the pathway leading to DNA synthesis?

4) if 2 is shown to be important for anchorage independent growth, does retinol interfere with secretion of matrix or adhesion proteins?

APPENDICES

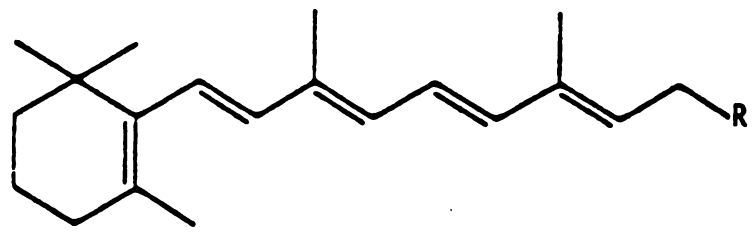
APPENDIX A

Definition of retinoids

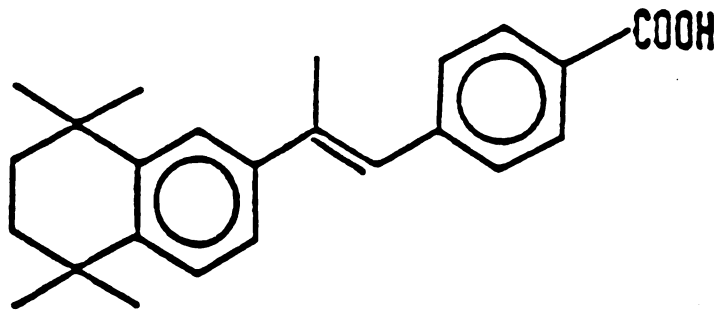
The International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) define retinoids as substances related to vitamin A (retinol). Chemically they are described as diterpenoids derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional group at the terminus of the acyclic portion (Figure 1). Classically, the functional group can be an aldehyde (retinal), alcohol (retinol), carboxylic acid (retinoic acid) or an acyl ester (retinyl palmitate or retinyl acetate). Sporn and Roberts (1985) and Sporn et al. (1986) have presented reasons for revising this definition. They note that vitamin A is not the ideal reference compound since many synthetic analogues are more potent in biological assays of vitamin A activity than retinol itself. One example is arotenoid or (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) which is neither a diterpenoid nor is it derived from a monocyclic compound containing five double bonds. The definition of a retinoid proposed by Sporn and Roberts, 1985, is "a substance that can elicit specific biological responses by binding to and activating a specific receptor or set of receptors. The classical ligands for these receptors are retinol and retinoic acid, but it is clear that synthetic ligands can have a better molecular fit to these receptors than

Figure 1 - Parent structure of retinoids and a synthetic retinoid. The structure shown in A is the parent compound of the substances referred to as retinoids. The structure shown in B is arotenoid.

A)



B)



retinol or retinoic acid. The programme for the biological response of the target cell resides in the retinoid receptor rather than in the retinoid itself." The new definition focuses on the receptor system to define the molecular mechanism of action of retinoids. While this new definition is more descriptive of the biological role of retinoids, it is not clear whether it will be generally accepted; therefore, I've used the classical definition in this dissertation.

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APPENDIX B

Anchorage Independent Growth

The ability of cells to form colonies in semisolid medium, anchorage independent growth, is a phenotype that is characteristic of tumor-derived cells. The alterations in a cell that enable it to exhibit this growth are not known. The anchorage indpendence assay technique has been used as a selective assay for virus-transformed cells (Macpherson, 1973) based on the ability of cells infected with a virus to form colonies in semisolid medium and the inability of uninfected cells to do so.

The selectivity of agar suspension culture has been attributed to the inhibitory effect of acidic and sulfated polysaccharides present in most agar (Macpherson, 1973). Other factors that affect the ability of cells to grow in semisolid medium are the concentration of serum and collagen (Macpherson, 1973). Increasing the concentration of serum and the addition of glucocorticoids to the medium increase the frequency of anchorage independent growth (Peehl and Stanbridge, 1981). Since serum contains peptide growth factors, it is thought that one or more of these growth factors may be responsible for anchorage independent growth. Several investigators have tested the ability of various growth factors to induce anchorage independent growth in rodent fibroblasts and we have tested them in human fibroblasts (chapter 2). Specific peptide growth

factors such as PDGF can induce anchorage independent growth in various cell types.

The effect that a peptide growth factor has on a cell that enables the cell to grow in semisolid medium is unknown. The stimulation of secretion of matrix or adhesion proteins such as fibronectin and collagen may be involved in anchorage independent growth. For example, Ignotz and Massague (1986) have reported that TGF-β stimulates the expression of fibronectin and collagen by normal rat kidney cells.

Even though the phenotype of anchorage independent growth is often used to quantitate the transformation of cells, very little is known about what changes occur in the cell to enable it to grow in semisolid medium.

References

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