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ONCOGENE TRANSFECTION STUDIES, IMMUNOHISTOCHEMICAL AND HISTOPATHOLOGIC EVALUATION OF TUMORS INDUCED BY ONCOGENES OR CARCINOGEN TREATMENT OF AN INFINITE LIFESPAN HUMAN FIBROBLAST CELL STRAIN.

By

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ABSTRACT

ONCOGENE TRANSFECTION STUDIES, IMMUNOHISTOCHEMICAL AND HISTOPATHOLOGIC EVALUATION OF TUMORS INDUCED BY ONCOGENES OR CARCINOGEN TREATMENT OF AN INFINITE LIFESPAN HUMAN FIBROBLAST CELL STRAIN

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Previous studies showed that transfection of an infinite lifespan, near-diploid, human fibroblast cell strain MSU-1.1, with vectors designed for high expression of the H- or N-*ras* oncogenes, caused the cells to form malignant tumors in athymic mice. Transfection of the same oncogenes in vectors that caused low levels of protein expression caused the MSU-1.1 cells to acquire some transformed properties but the cells were non-tumorigenic. This study examined the effects of various expression levels of the H-*ras* oncogene on malignant transformation of the human fibroblast cell strain MSU-1.1. A plasmid which carries the H-*ras* oncogene under the control of the metallothionein promoter (MT) was used to transfect MSU-1.1 cells. Transfection and expression of this plasmid in MSU-1.1 cells, caused the cells to be transformed because the H-*ras* oncogene was

upregulated when zinc was added to the cell culture medium. These MT-*ras* transformed cells formed tumors in athymic mice given zinc-supplemented drinking water. Transfer of tumor-bearing mice to non zinc-supplemented drinking water had no effect on the growth pattern of tumors. Malignant tumors were produced by the transformed cells that expressed the H-*ras* oncoprotein at high levels and benign tumors were produced by transformed cells that expressed the H-*ras* oncoprotein at lower levels. These data indicate that a critical level of expression of the H-*ras* oncogene is required for malignant transformation of MSU-1.1 cells.

In another study (±)-7ß, 8α-dihydroxy-9α,10α,-epoxy-7,8,9,10-tetra-hydrobenzo[a] pyrene (BPDE) transformed cells formed malignant tumors that exhibited increased expression of the *ras* protein whereas low grade spindle cell sarcoma and fibromas did not. The tumors formed by oncogene or carcinogen transformed MSU-1.1 cells from current and previous studies were evaluated morphologically. Seven distinct histologic patterns were identified and these patterns were similar to those used to describe soft tissue tumors in humans and animals. Therefore, this model of *in vitro* transformation of MSU-1.1 cells that form tumors should be useful in studying the mechanisms of soft tissue tumorigenesis in humans.

This dissertation is dedicated to my wife Victoria, our children Calvert Jr. and Tanise, a dear aunt Millicent Louden, my mother Ermine Louden and my late father, mentor and friend Melville Robinson Louden, whose wisdom, inspiration and love made it all possible.

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KEY TO ABBREVIATIONS

AAF	2Acetylaminofluorene
AI	Anchorage Independent
BPDE	±7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetra-hydro-benzo[a]pyrene
DEN	Diethylnitrosamine
DES	Diethylstilbestrol
DMBA	Dimethylbenz[a]anthracene
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ENU	Ethylnitrosourea
FAP	Familial adenomatous polyposis
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
^{3}H	Tritiated thymidine
MMNG	N-Methyl-N'-nitro'N-nitrosoguanidine
MNU	N-nitro N-methylurea
NK	Natural killer cells
4NQO	4 nitroquinoline-1-oxide
PH	Partial hepatectomy

PI	Phosphoinositide
PLC	Phospholipase C
Rb	Retinoblastoma
RNA	Ribonucleic Acid
SCS	Supplemented calf serum
SHE	Syrian hamster embryo
TGF	β Transforming growth factor beta
TPA	12-0-tetradecanoylphorbol-13-acetate

INTRODUCTION

Within the past three decades, the emphasis on cancer research has shifted from the individual to the environment. This in part is due to the strong evidence that implicates the environment as a major factor in the pathogenesis of human cancer (Higginson, 1969). Although many factors have contributed to this shift, one of the most significant is the general acceptance of cigarette smoking as a major contributor to the increasing incidence of lung cancer (Doll, 1980).

While the contributory role of tobacco to the pathogenesis of some types of human cancer is well recognized, the available research data indicates that many different environmental factors play a causal role in inducing cancer. These include diet, exposure to radiation and chemical carcinogens. The largest of these groups by far are chemicals, both man made and those that occur naturally.

With the understanding of the metabolism of chemical carcinogens, and the nature of their active forms, the qualitative correlation between mutagenicity and carcinogenicity has become apparent (Miller and Miller, 1971). For example, Maher *et al.* 1968 reported that reactive derivatives of the carcinogens, 2-acetylaminofluorene (AAF) and N-methyl-4-amino-azo-benzene, have a high mutagenic activity in a DNA-transformation system, suggesting that such agents may cause cancer by inducing mutations.

A major recent advance in cancer research has been the development of *in vitro* assays for assesing cell transformation. These *in vitro* cell transformation assays have allowed analysis of the carcinogenic process in detail. For example, selection, isolation and characterization of transformed cells (i.e., cells with similar properties to cancer cells) can be accomplished *in vitro* after normal cells are either infected with certain viruses and/or treated with direct acting carcinogens or the reactive derivatives of those carcinogens that first need to be enzymatically metabolized.

Analysis of the multiple steps in carcinogenesis is further made possible by the development of molecular techniques such as DNA transfection, immunoprecipitation, immunohistochemistry, Southern, Northern, and Western blotting which allows investigators to detect specific gene and/or gene products within cells. Using these techniques researchers have investigated the interaction between the environment and genetic factors in the development of cancer.

Another important advance in cancer research has been the discovery of a unique class of cellular genes (oncogenes) that when mutated in specific ways, cause normal cells to "take on" phenotypic properties associated with cancer cells, i.e., to become transformed (Knudson, 1985). A cell that acquires an appropriate combination of such changes is neoplastically transformed, i.e., it can form a tumor in an appropriate host. Oncogenes are altered, dominantly-acting, or inappropriately expressed forms of a class of cellular genes referred to as proto-oncogenes. The results of *in vitro* rodent cell transformation studies indicate that oncogenes play a causal role in human

(Krontiris and Cooper, 1981; Shih *et al.*, 1981) and animal carcinogenesis (Varmus, 1984). An equally significant finding was the discovery of tumor suppressor genes (Bienz *et al.*, 1984; Friend *et al.*, 1986). Typically, tumor suppressor genes act to regulate cell proliferation, and the loss of a functional suppressor gene causes the cells to gain tumor-cell like properties. The widespread occurrence of oncogenes (e.g., *ras*) and loss of tumor suppressor genes in human tumors form the basic framework for many current investigations on the development of cancer (Harris, 1992).

The neoplastic transformation of cells *in vitro* is generally considered to be an acceptable model of tumor formation *in vivo*. In spite of the considerable evidence demonstrating that carcinogen exposure is one of the primary causes of human cancer and that carcinogens have the ability to transform several non-tumorigenic rodent cell lines into malignant cells; until recently, normal human cells in culture had never been successfully transformed to malignancy by exposure to carcinogens (McCormick and Maher, 1988). Since malignant transformation is now recognized as a multi-step process, a possible explanation for the failure of researchers to obtain malignantly-transformed cells in culture was their inability to recognize the intermediate phenotypic changes exhibited by carcinogen-treated cells (McCormick and Maher, 1988). To examine this hypothesis McCormick and Maher showed that transfection of finite life span, diploid, human fibroblasts with oncogenes known to be activated in human fibrosarcomas, or treatment of these diploid human fibroblasts with carcinogens, caused transfected or carcinogen treated cells to form foci, become morphologically

transformed and anchorage independent (McCormick and Maher, 1988). However, these transformed cells did not induce tumors when injected subcutaneously in athymic mice and all had a finite life span in culture which allowed only two successive clonal selections before the cells senesced. From these experiments they concluded that in addition to the phenotypes expressed by these transformed cells acquisition of an infinite or greatly extended life span might represent a critical intermediate step in the process of malignant transformation. If this hypothesis was correct, to study the process of malignant transformation of human fibroblasts, it would be ideal to have a diploid, human fibroblast cell line that exhibits an infinite life span, but is otherwise completely normal. Although the existence of such a cell line had never been described, McCormick, Maher and their colleagues were successful in developing such a strain after transfection of a normal, diploid, human fibroblast cell line with the v-*myc* oncogene (Morgan *et al.*, 1991). A clonal population of cells expressing the v-*myc* protein was isolated and passaged until senescence. Among the senescing cells, viable cells gave rise to a cell strain they called MSU-1.1. Karotypic analysis indicated that these cells were clonally-derived, with 45 chromosomes including 2 marker chromosomes. When an early passage of post senescing cells were karotyped, a diploid population of cells designated MSU-1.0 was identified. MSU-1.0 is also an immortal cell strain. The evidence suggests that the MSU-1.1 strain is a spontaneous derivative of MSU-1.0 strain.

The MSU-1.0 cells grow only in medium with exogenous growth factors; the MSU-1.1 cells grow at a moderate rate in medium without exogenous growth factors (Morgan *et al.*, 1991). In later experiments, MSU-1.1 cells transfected with vectors carrying the H- or N-ras oncogene and designed for high expression of these oncogenes, caused the transfected cells to form foci of transformed cells. Cells isolated from these foci were morphologically transformed, exhibited anchorage independent growth and grew in medium lacking exogenous growth factors (Hurlin *et al.*, 1989; Wilson *et al.*, 1990). Similar results were achieved using the v-Ki-*ras* (Fry *et al.*, 1990) oncogene and more recently the v-*sis* oncogene (Yang *et al.*, 1994). All of these ras-transformed cells formed rapid-growing tumors when injected into athymic mice (Hurlin *et al.*, 1989; Wilson *et al.*, 1990; Fry *et al.*, 1990).

Using the *in vitro* characteristics induced by *ras* transformation (focus formation and growth in agarose) Yang *et al.*, (1992) were able to malignantly transform MSU-1.1 cells by treatment with (±)-7ß,8α-dihydroxy-9α, 10α-epoxy-7, 8, 9, 10-tetrahydrobenzo [a] pyrene (BPDE). Similarly, treatment of MSU-1.1 cells with the carcinogens ethylnitrosourea (ENU), ICR-191, or ionizing (Reinhold *et al.*, 1996 radiation caused the cells to form foci of morphologically altered cells and the cloned progeny induced tumors in athymic mice (McCormick, JJ unpublished studies). This dissertation represents an expansion of previous studies carried out on the mechanisms involved in the malignant transformation of the MSU-1.1 cells.

The *ras* family of oncogenes has been identified in many different types of human tumors (Eva *et al.*, 1982; Bos *et al.*, 1987; Bos *et al.*, 1985) and have been the subject of extensive research. As indicated above, the H-*ras* (Hurlin *et al.* 1989) and the N-*ras* (Wilson *et al.*, 1990) oncogenes can transform MSU-1.1 cells to malignant cells. Recent studies show that when the same oncogenes are expressed at much lower levels, they do not transform these cells to malignancy (McCormick, JJ unpublished studies). This finding indicates that there is some critical level of *ras* oncoprotein expression that is required for malignant transformation of the MSU-1.1 cells. To address the question as to how much ras expression is required for malignant transformation I used a vector in which the H-*ras*T24 oncogene was placed under the control of a promoter that can be modulated by the zinc concentration of the culture medium or the serum of animals, and: (1) determined the relationship between expression levels of the H-*ras* oncogene and malignant transformation *in vitro*, (2) determined if different levels of expression of the H-*ras* oncogene *in vivo* controls the tumor phenotype, (3) determined the effect of down regulation of expression of the H-*ras* oncogene on the biological behavior of H-*ras* induced tumors. The results indicated that malignant transformation of the H-*ras* oncogene transfected MSU-1.1 cells *in vitro* and *in vivo* is determined by the level at which the *ras* oncogene is expressed. The results of that study forms **Chapter II** of this dissertation. It is written in the style and format of **Cancer Research** so that the paper can be submitted to this journal for publication.

Chapter III which is also written in the style and format of papers to be published in the journal **Cancer Research** summarizes an immunohistochemical study I carried out to determine if there is an increased expression of the *ras* protein in tumors induced by cloned cells obtained from transformed foci which occurred as a result of treatment of MSU-1.1 cells with the carcinogen BPDE. The results indicated that high grade malignant tumors show a strong positive immunoreactivity to a pan *ras* monoclonal antibody but that benign and low grade malignant tumors show no staining reaction when they are stained with the same antibody.

The findings from the above studies as well as others formed the basis for a comprehensive review of the morphology and pattern classification of tumors induced by MSU-1.1 transformed cells that were derived from oncogene transfection studies and/or carcinogen treatment of MSU-1.1 cells. I carried out this study in collaboration with Dr. Robert Dunstan of the Department of Pathology and Drs McCormick and Maher of the Carcinogenesis Laboratory. The primary objectives of the study were to determine if the experimentally-induced tumors were similar morphologically to soft the tissue tumors found in humans and animals. I also examined the relationship between tumor type, grade of malignancy and the expression of specific oncogenes. The results of this study showed that independent transformed cell strains derived from a single cell strain (MSU-1.1) induced tumors which exhibited a wide range of phenotypes. These phenotypes resemble those found among many of the common soft

tissue tumors seen in humans. The data indicate that transformed cells induced by carcinogen treatment and/or oncogene transfection of MSU-1.1 cells can be used to study the genetic changes involved in the development of human soft tissue tumors. These findings are summarized in **Chapter IV** and are written in the style and format for papers submitted of the "**American Journal of Pathology**" so that the paper can be submitted to this journal for publication.

CHAPTER I

LITERATURE REVIEW

IMPORTANT HISTORICAL EVENTS IN CARCINOGENESIS

The beginning of our knowledge of chemical carcinogenesis can be traced to the observations of two London physicians, John Hill and Percival Pott. In 1761 Hill reported a high incidence of nasal cancer as a consequence of excessive use of tobacco snuff (Redmon, 1970). In 1775 Pott reported on the high incidence of scrotal cancer in chimney sweeps (Pott, 1775). He suggested that the soot, to which the workers were exposed, was caught in the folds of the scrotal skin and caused cancer. Pott prescribed more frequent baths to remove the soot from the scrotal skin folds, and this behavioral change reduced the incidence of scrotal cancer. A hundred years later Volkmann, a German physician, recognized exposure to tar as a cancer-stimulating principle. However, it was not until 1918 that two Japanese workers, Yamagiwa and Ichikawa, succeeded in the experimental induction of skin tumors by the application of coal tar followed by scarlet oil to the ears of rabbits. This led to experiments by many workers (e.g., Rous and Kidd, 1941; Mottram, 1944; Berenblum and Shubik, 1947) to analyze this carcinogenic process. From these experiments, the initiation-promotion concept arose. This led to the development of initiation-promotion procedures for the induction of tumors in the skin, liver, mammary gland, lung, stomach and colon.

Initiation

If one interprets the initiation-promotion concept in modern terms, initiation is the first event in chemical or radiation carcinogenesis. It occurs as the result of interaction

of radiation or a chemical carcinogen (or its metabolite) with specific cancer-related gene(s) in the DNA of the host cell, which leads to mutations in this gene, and causes the cells with this change to express a tumor-like property. Schulte-Hermann (1985) defined initiation as an "irreversible, heritable, cellular alteration caused by a carcinogen which creates a potential in a cell and its progeny, for subsequent malignant transformation." From the studies of Kakunaga (1974) and Columbano *et al.* (1981) it was clear that cell proliferation was required for "fixing" (i.e., making permanent in some way) of the carcinogen induced damage for the successful development of transformed cells. These findings are in agreement with earlier reports that most carcinogens or the metabolites of chemical carcinogens cause mutations in DNA (Maher *et al.*, 1968). In the process of DNA replication, carcinogen adducts in DNA causes misreplication leading to a change in the DNA sequence i.e., a heritable mutation. Presumably DNA replication is the "fixation" event. It is now widely accepted that an appropriate mutation in a cancer-related gene creates an initiated cell. The initiated cells commonly have a selective growth advantage causing them to proliferate at a higher rate when compared to their sister cells (Gunnar and Seglen, 1990). The subsequent malignant transformation of initiated cells may occur with the passage of time and without further treatment with exogenous agents, presumably, through additional spontaneous mutations, but the process is strongly encouraged by exposure to promoting agents (Schulte-Hermann, 1985).

Promotion

In classical cancer studies, the term promotion is used to define the process of proliferative expansion of a population of initiated cells caused by repeated application of the promoting agent (Schulte-Herman, 1985). When the application of the tumor promoting agent is stopped before malignant tumors arise, the effect can be completely reversible, i.e., the cells return to a normal phenotype, whereas in other cases, the cells continue to exhibit the transformed phenotype. For example, in the mouse skin model of carcinogenesis, papillomas regress completely after application of the promoting agent has ceased. In the liver, some focal lesions (foci) regress when application of the promoting agent has ceased. Other foci remain and a small number of these may develop to form tumors.

A very significant advancement in the understanding of tumor promotion was the finding that the common tumor promoter *12-O*-tetradecanoylphorbol-13-acetate (TPA) an analog of diacylglycerol, leads to activation of protein kinase C (Castagna *et al.*, 1982). The activation of protein kinase C initiates a cascade of intracellular events that can result in increased cell proliferation. Other authors have suggested that tumor promoters have additional modes of action. For example, Yuspa and Morgan (1981) reported that TPA also induces the resistance of initiated cells to terminally differentiate. Other studies indicate that initiated-promoted cells have lost their ability to respond to negative growth regulators such as transforming growth factor-B-1

(TGF-ß) (cf Gunnar and Seglen, 1990; Parkinson, 1985). It is not clear if a similar effect takes place when cells in culture are treated with promoting agents such as TPA.

Progression

In classical tumor studies the term progression is used to describe the spontaneous process of transformation of initiated cells to cells with the tumorigenic phenotype (benign or malignant) with increasing degrees of anaplasia in the malignant tumors (Schulte-Hermann, 1985). Progression is irreversible. The critical events in this stage are now understood to be additional genetic changes that are required for tumor development.

MORE RECENT INSIGHTS: MULTI-STAGE CONCEPT AND GENETIC BASIS OF CARCINOGENESIS

Early experiments by Friewald and Rous (1944) and Berenblum and Shubik (1947) using the rabbit and mouse skin models of carcinogenesis respectively, indicated that cancer development is the result of a multi-stage process. Their results suggested that the administration of a limited dose of a chemical carcinogen (initiator) to rabbit or mouse skin causes changes in some cells, that are imperceptible in the absence of promoter treatment, and do not by themselves result in tumors. Later studies confirmed these findings in that administration of only a single small dose of an initiator or only repetitive doses of a promoter does not lead to gross tumors (Boutwell, 1964; Loveless, 1969; Vann Duuren, 1976). However, administration of the initiator followed by repeated doses of the promoter gave rise to a large number of

papillomas (benign tumors) in a short period and carcinomas (malignant tumors) after a long latency (1 year). Although initiation occurs after only a single treatment with the appropriate concentration of a carcinogen, tumors develop only if the promoter is applied many times. However, many months can elapse between application of the initiator and the promoter (Berenblum, 1949; Van Duuren *et al.*, 1975)). No tumors form if the promoter is given before the initiator. Since these early studies on mouse and rabbit skin carcinogenesis, the multi-step nature of the process has been demonstrated in liver (Periano *et al.*, 1973) and mammary gland (Armuth and Berenblum, 1972) using a similar approach. These results clearly suggest that multiple stages are involved in tumor formation and can be explained as follows: Initiation causes a cell to acquire some change(s) associated with tumor-derived cells that confers a growth advantage. By clonal expansion, numerous progeny cells arise exhibiting that phenotype. This greatly increases the chances that one of these initiated cells will acquire an additional change related to carcinogenesis (McCormick and Maher, 1988). The process continues until malignant cells arise.

Epidemiological studies also support the multi-stage theory of cancer development. The data from these studies indicate that tumor development is the end product of a series of independent events in a cell, the exact number of which depends on the particular type of target cell (Doll, 1980).

Oncogenes and Proto-oncogenes

In the last few years significant progress was made in understanding the molecular basis of carcinogenesis. This was due in part to the identification of oncogenes and proto-oncogenes. Proto-oncogenes are cellular genes commonly associated with processes such as cell growth, proliferation and differentiation. These genes are well-regulated and are expressed at specific times and frequently in a tissue specific manner (Bishop, 1987). In contrast, oncogenes are mutated forms of proto-oncogenes or proto-oncogenes with altered expression. Oncogenes play a causal role in cell transformation.

Discovery

One of the most significant recent advances in cancer research was the discovery that some human cancer cells have permanent heritable changes in specific genes. Weinberg and his colleagues (Shih *et al.*, 1981), Cooper *et al.*, (1980), and Krontiris and Cooper (1981) transferred DNA from human tumor cell lines to NIH 3T3 cells, an infinite life span mouse fibroblast cell line. The transfected cells formed foci and grew in soft agarose. Agar colonies and foci isolated cells formed progressively growing invasive tumors when injected into athymic mice. In contrast, DNA from normal human cells did not cause NIH 3T3 cells to form foci or grow in soft agarose and the transfected cells were not transformed or tumorigenic. The fact that the direct introduction of distinct fragments of human tumor-derived DNA, into cells caused

malignant transformation provided the first evidence for the presence of dominantly acting cancer genes in human tumors but not in normal human cells.

Mechanisms of Proto-oncogene Activation.

The concept of oncogenes is supported by studies in which it was demonstrated that activation of proto-oncogenes to oncogenes can occur by capture of proto-oncogenes by RNA tumor viruses, as in the case of acute transforming retroviruses. Other mechanisms of proto-oncogene activation includes insertional mutagenesis, gene amplification, point mutations, gene rearrangements and chromosomal translocation (Weiss *et al.*, 1982; Duesberg, 1983). Some of these will be discussed briefly.

Oncogenes in Acute Transforming Retroviruses

Acute transforming retroviruses cause rapid induction of tumors. The first such virus was identified in chickens by Rous (Rous, 1911). He reported that a non-cellular fraction induced a sarcoma identical to the sarcoma from which the fraction was isolated. Later studies with this virus led to the discovery of the first oncogene *src*, by Bishop and colleagues (Stehelin *et al.*, 1976). The virus also transforms fibroblasts *in vitro* (Tooze, 1973; Vogt, 1969). Since those early studies other oncogenes have been identified in acute transforming viruses. For example, one form of acute leukemia in chickens is caused by Avian Erythroblastosis virus. This virus carries the v-*erb* oncogene (Graft and Beug, 1983). Some viral strains transform fibroblasts *in vitro* (Pokora-Royer *et al.*, 1978) and induce sarcomas in chickens (Yamamoto *et al.*, 1983). It is now known that acute transforming retroviruses carry discrete segments

of host DNA captured by the virus during infection of host cells. Recombination causes host genetic material to be inserted in place of viral replicative genes. The viral promoters can cause overexpression of the "captured gene" and the increased expression of the gene product confers the transforming ability on these viruses (Aaronson, 1982). In acute transforming retroviruses, the captured host genetic material is referred to as a viral oncogene.

Activation of Proto-oncogenes by Slow Transforming Viruses

In contrast to acute transforming retroviruses, slow transforming retroviruses do not carry oncogenes (Duesberg, 1983). Many of these viruses cause leukemia in rodents and, so they have often been referred to as chronic leukemia viruses. Hayward *et al.*, (1981) and Payne *et al.*, (1981) discovered that these viruses can cause neoplastic transformation by insertional mutagenesis. Slow transforming viruses infect cells and integrates randomly within the genome of the infected cell. If the integration site is 5' to a proto-oncogene, the presence of the 3' viral promoters may cause increased transcription of the proto-oncogene. In some circumstances the increased expression is sufficient to activate the proto-oncogene. Some proto-oncogenes that have been found to be activated by insertional mutagenesis include c-*mos*, c-*myc*, c-*myb* and H-*ras* (cf., Varmus, 1984).

Activation by Gene Amplification

Amplification of a gene, i.e., increasing the number of copies, can lead to increased protein production of the gene (Alitalo and Schwab, 1983). For example, many

neuroblastomas contain multiple copies of the N-*myc* oncogene (Kohl *et al.*, 1983; Schwab *et al.*, 1983a; Brodeur *et al.*, 1984). The presence of multiple copies of the N-*myc* gene usually increases the steady-state levels of the N-*myc* protein in cancer cells with this change. The evidence suggests that in neuroblastomas with this change the increased level of N-*myc* protein causes the uncontrolled growth of these cells with this genetic change. Another example of gene amplification occurs in human mammary carcinomas. Burbeck *et al.*, (1984), Libermann *et al.*, (1985), and King *et al.*, (1985) found that EGF-receptor-related sequences were amplified in some cases of human mammary carcinoma. The higher number of receptors are thought to allow a greater number of growth factor proteins to enter a cell. It is postulated that this drives cell replication at a higher rate than that of cells with fewer receptors. There are a number of other reports of oncogene amplification in the tumorigenic process. For example, the K-*ras* and *abl* proto-oncogenes, respectively, are amplified in mouse adrenocortical tumor (Schwab *et al.*, 1983b) and in chronic myelogenous leukemia (Collins and Groudine, 1982a).

Activation by Chromosomal Translocation

More than 60 proto-oncogenes have been identified in the human genome (Cotran *et al.*, 1989). In 1981, Kline hypothesized that proto-oncogenes could be activated by chromosomal translocation. Tumors which exhibited a high frequency of a specific chromosomal translocation pattern were suggested to have an oncogene activated by this mechanism. Specific evidence in favor of this hypothesis was presented by Dalla

Favera *et al.*, (1982a) and Taub, *et al.*, (1982) who showed that in Burkitt's lymphoma the c-*myc* gene is translocated from chromosome 8 to chromosome 14. This brings the c-*myc* gene in proximity to an immunoglobulin gene which has a high transcriptional activity in B-lymphocytes (Ar-Rushidi *et al.*, 1983). The c-*myc* gene is thus subject to regulation by the active immunoglobulin promoter. At this new location the c-*myc* gene is constitutively expressed at a high level and it is generally accepted that this increased expression plays a role in the etiology of this tumor.

Chronic myelogenous leukemia is another example in which chromosomal translocation contributes to the cause of human cancer. In this disease a unique marker chromosome referred to as the Philadelphia chromosome is seen (DeKline *et al.*, 1982). The Philadelphia chromosome consists of the 5' region of *abl* gene on chromosome 9 which is fused to the *bcr* gene on chromosome 22 and in the process of recombination the *abl* gene is rearranged. This results in the expression of a *abl bcr* fusion protein (Shtivelman *et al.*, 1985) which exhibits a higher level of tyrosine phosphorylase than the c-*abl* proto-oncogene product. This increase in tyrosine phosphorylation activity contributes to cellular transformation in chronic myelogenous leukemia (Davis *et al.*, 1985). Thus the *abl* gene becomes an oncogene by two mechanisms: (1) aberrant expression of *abl* transcripts, (2) structural change (genetic rearrangement) in the gene resulting in an altered protein.

Activation by Point Mutation

There are several proto-oncogenes known to be activated to oncogenes by point mutations. The best characterized is the *ras* gene. The genetic lesions (mutations) responsible for the activation of *ras* proto-oncogenes have been localized to single base pair changes in the coding sequence of these genes. The mutations lead to single amino acid substitution in the 21kd *ras* protein (p21). Point mutations within the coding sequences of the *ras* genes most commonly affect codons 12 or 61 and to a lesser degree codons 13 and 59 (cf Spandidos, 1989). The aberrant protein produced by the mutant *ras* gene have been shown to have transforming ability (Ferminisco *et al.*, 1985; Spandidos and Wilkie, 1984a; Hurlin *et al.*, 1989; Wilson *et al.*, 1990).

In addition, point mutations within one of the introns of the *ras* gene can cause the gene to be overexpressed leading to increased ras protein production (Cohen and Levinson, 1988). Increase in *ras* protein production has been shown to contribute to the development of human cancer in some cases (Tabin *et al.*, 1982; Santos *et al.*, 1983; Suarez *et al.*, 1987).

In summary, proto-oncogenes can be activated to oncogenes by different mechanisms. Irrespective of the mechanism of proto-oncogene activation, the end result is that there is a change in the primary DNA structure, i.e., mutation. The protein produced as a result of these changes typically cause increased cell proliferation.

Activation of Proto-oncogenes in Chemical Carcinogenesis

Mutant forms of the *ras* genes have been repeatedly identified in cells from a significant number of tumors from humans and animals (cf., Spandidos and Lang, 1989). In addition, *in vivo* carcinogenicity studies frequently report that cells from chemically-induced tumors have mutations in the *ras* gene (cf., Guerrero and Pellicer, 1987). Chemical carcinogens can interact with DNA causing activation of the *ras* proto-oncogene by point mutations and/or overexpression. The end result of this activation is the production of an abnormal protein and /or increased *ras* protein synthesis that causes increased cell proliferation which plays a causal role in carcinogenesis (Ebert *et al.*, 1990).

Chemical carcinogens that have been reported to cause mutations in the *ras* gene include, NMU, (Newcomb *et al.*, 1989), DMBA, (Quintanilla *et al.*, 1986), DEN, (Stowers *et al.*, 1988), MMNG and BPDE (Stevens *et al.*, 1988). DNA sequence analysis shows that point mutations in codons 12 and 61 are most frequent (Barbacid, 1987) with less frequent involvement of codons 13 and 117 (Bos *et al.*, 1985; Reynolds *et al.*, 1987).

Another typical proto-oncogene activated in chemically induced tumors is the *myc* gene (Cooper, 1991). For example Ebert *et al.*, (1990) reported that trenbolone transformed SHE cells exhibited an increase in *myc* protein expression. Several studies suggest that the *myc* oncogene can participate in the tumorigenic process in several ways. Bishop and colleagues (Schwab *et al.*, 1985) and Yancopoulos *et al.*,

(1985) reported that the *myc* oncogene can cooperate with a mutant H-*ras* proto-oncogene to transform normal embryonic rat cells in culture. They also concluded that abnormal expression of the *myc* oncogene plays a causal role in tumor development or progression by causing an extension of the lifespan (immortality) of the transfected cells in culture. Acquisition of the immortal phenotype has been suggested to be a necessary step for development of malignant tumors (McCormick and Maher, 1988). Studies by Kaczmarek *et al.*, (1985) and Cole (1986) indicate that the *myc* gene encodes for a nuclear protein that is involved in DNA replication and causes quiescent cells to begin DNA synthesis which leads to increased cell proliferation. However, the work of Prochownik and Kubowska (1986) and Larsson *et al.*, (1986) indicate that overexpression of the *myc* protein or the inability of cells to down regulate this protein may cause them to lose their ability to undergo terminal differentiation. The body of available research data indicates that the *myc* gene functions as an oncogene in human tumors when it is expressed at higher than normal levels (Andeol *et al.*, 1988).

TUMOR SUPPRESSOR GENES

Tumor suppressor genes are the other major class of genes which have been found to play a causal role in carcinogenesis (Klein, 1987; Knudson, 1985). In contrast to proto-oncogenes, tumor suppressor genes play a causal role in carcinogenesis when cells lose the ability to make functional protein product. Since these genes have not been found on the X or Y chromosomes, loss necessarily involves independent genetic changes for both copies of such a gene. Typical changes are homozygous deletion of

the gene, small or large deletions, splicing mutations (Horowitz *et al.*, 1990) base pair changes and small deletions in the promoter region (Bookstein *et al.*, 1990a).

Discovery

There are three major lines of experimental evidence that support a role for tumor suppressor genes: studies on cell hybrids, analysis of familial cancers and analysis of loss of genetic heterozygosity in tumors. One of the first studies on tumor suppression was reported by Weissman and Stanbridge (1983). They found that fusion of one type of tumor cell to another type of tumor cell sometimes resulted in hybrid clones that were not tumorigenic. They hypothesized that these tumor cells each had lost a different tumor suppressor gene (s) in the process of carcinogenesis and when fused were able to complement the defects of each other and formed non-tumorigenic hybrid clones. In a 1988 study, Harris found that the fusion of tumorigenic and non-tumorigenic cells resulted in cell hybrids that were not tumorigenic. He hypothesized that: (1) a tumor suppressor gene limits the growth of the non-transformed cell, (2) loss of the function of this gene plays a causal role in the tumorigenic phenotype of the transformed cell, (3) the hybrid cells, are not tumorigenic because the non-transformed cell has complemented the defect of the transformed cell. In this interpretation, loss of a tumor suppressor gene is causally involved in cancer development.

The identification and characterization of tumor suppressor genes have come primarily from studies on familial cancer with the ideas of Demars (1969) and

Knudson (1971) serving as a framework. Familial cancers in which tumor suppressor genes have been identified include retinoblastoma, neurofibromatosis, Wilms' tumor, familial adenomatosis polyposis (FAP) and multiple endocrine neoplasia. The suppressor genes best characterized are the RB gene of retinoblastoma and the P53 gene which has been associated with many types of human tumors. Some important features of these two genes will be discussed briefly followed by a brief discussion of other suppressor genes.

Retinoblastoma Gene (RB)

Retinoblastoma is the most common malignant eye tumor of childhood and occurs in familial and non-familial (sporadic) forms. Children with the familial form typically develop multiple, bilateral retinal tumors by age three or four (Robbins, Kumar and Cotran 1989). Children that survive the retinal tumors are at a high risk for other tumors later in life. Francke (1978) showed that there was a deletion in one copy of chromosome 13 at 13q14 in all somatic cells from some patients with hereditary retinoblastoma. The genetics of familial retinoblastoma can be explained as follows: an individual inherits a mutant allele from an affected parent and through a somatic event the other normal allele becomes inactivated (cf., Marshall, 1991).

In contrast, in the sporadic form of retinoblastoma tumors are unilateral and focal and there is no increased risk for other tumors. The genetics of sporadic retinoblastoma can be explained as follows: an individual with sporadic retinoblastoma inherits two normal alleles for the RB gene. One becomes inactivated in one of the

cells that give rise to the retina in one eye, so that all or a sizable population of the retinal cells of one eye carry a mutation in the RB gene. A second inactivating mutation in the RB gene occurs in the descendants of the cell that received the first mutation (cf., Marshall, 1991). It is clear from this, that retinal cells that give rise to non-hereditary retinoblastoma must acquire two somatic mutations in a single cell, and since the somatic mutation rate is low these patients will have only a single focus of tumor.

Friend *et al.*, (1986) showed that there was homozygous deletion on chromosome 13 (mapped to the 13q14) in normal retinoblasts and retinoblastoma tumor-derived cells from some patients with hereditary RB. This model shows that the retinoblastoma gene may be homozygously deleted in which case the RB gene could not be expressed in retinoblasts from the normal retina or in retinoblastoma. In the same study Friend *et al.*, (1986) showed that some retinoblastoma cells had internal deletions within the RB gene which presumably cause inactivation of the gene. The loss of suppressive function of the RB gene can also occur through other genetic events. These events include: large scale deletions within the gene, splicing mutations which result in deletion of an Exxon (Horowitz *et al.*, 1989) point mutations (Kaye *et al.*, 1990), and small deletions in the promoter region (Bookstein *et al.*, 1990a).

From the studies described it is clear that the RB gene functions as a tumor suppressor gene. The suppressor function of this gene is lost when damage to the genetic material results in loss of the gene, (deletion) or the production of an abnormal

protein due to a mutation. In either circumstance, the suppressive, negative-growth regulatory function of the Rb protein is lost and this leads to increased cell proliferation.

Lee *et al.*, (1987) showed that the RB gene encoded a 928 amino acid protein, p105-RB, that is expressed in retinoblasts as well as other tissues. Horowitz *et al.*, (1990) used immunoprecipitation to confirm earlier findings that all short term cultures of retinoblastoma cells showed no expression of the RB protein (p105RB). Recent studies have shown that a variety of different tumors contain inactivated RB alleles. These include sporadic sarcomas, sarcomas arising from families with retinoblastoma (Cooper and Stratton, 1991), small cell lung carcinomas (Harbour *et al.*, 1988), breast carcinomas (Lee *et al.*, 1988; T'Ang *et al.*, 1988) and about a third of urinary bladder cancer cell lines (Horowitz *et al.*, 1990). The data from these studies indicate that loss of function of the RB protein either through lack of expression, decreased expression or production of an abnormal protein, plays a role in tumor development of many tumors.

The work of Harlow and collaborators (Whyte *et al.*, 1988) provided a significant insight into the mechanism of action of the RB protein. They showed that the p105-RB complexed to the transforming protein of adenovirus. This interaction is essential for the transformation of cells by adenoviruses. In another study DeCaprio *et al.*, (1988) showed that the p105-RB also binds to SV40 Large T antigen. Ludlow *et al.*, (1989) and DeCaprio *et al.*, (1988) reported that the binding to Large T antigen is

regulated by phosphorylation and only the hypophosphorylated form of the RB protein can suppress cell proliferation. These findings suggest that the transforming proteins of adenovirus and SV40 act by complexing the hypophosphorylated RB protein which inactives it (cf Marshall, 1991). Data to support this finding comes from the work of Kaye *et al.*, (1990) and Templeton *et al.*, (1991) who showed that tumor-derived cells with a specific point mutation in the RB gene produce a protein that cannot undergo dephosphorylation.

Based on these studies and others the RB protein is thought to control normal cell growth in the following manner: Normal cells in G_0 produce an RB protein which is hypophosphorylated. The hypophosphorylated form blocks cell proliferation i.e., acts as a growth suppressor. As a result of exposing cells to an appropriate growth factor, the RB protein becomes hyperphosphorylated and this protein no longer suppresses cell proliferation. A similar effect is seen when there is deletion of the RB gene, because the normal RB protein which usually suppresses cell proliferation is lost.

P53 Gene

A region on the short arm of chromosome 17 was shown to be lost frequently in colon carcinomas (Baker *et al.*, 1989). This site was later found to contain the p53 gene. Loss of p53 gene expression has also been reported in many types of cancer. For example, Mowat *et al.*, (1985) observed that Friend virus transformed, leukemic cells had insertions or deletions at the p53 locus which resulted in complete loss of expression of the p53 protein in the transformed cells. It was hypothesized that when

functional, the p53 gene suppresses cell growth and that loss of function of this gene contributes to uncontrollable proliferation and therefore tumorigenesis. An example of this is seen in the study by Bookstein *et al.*, (1990b) in which the tumorigenicity of a prostatic carcinoma cell line which have a mutant p53 gene was suppressed when the non-mutated normal p53 gene was placed in these cells. Further evidence to support the tumor suppressor function of the p53 gene comes from the work of Finlay *et al.*, (1989) and Eliyahu *et al.*, (1989). They showed that transfection and expression of normal p53 could suppress some transformed phenotypes such as focus formation in cells previously transformed by mutant p53 or the *ras* oncogene. In a similar study, Zambetti *et al.*, (1992) demonstrated that normal p53 was able to suppress transformation induced by mutant p53 protein together with the *ras* oncogene. In a recent study, Shaw *et al.*, (1992) reported that the re-introduction and expression of the wild type p53 gene in a colonic carcinoma cell line with a deletion of the p53 gene, caused regression of tumors induced in athymic mice by this tumorigenic cell line.

cDNA sequencing and immunocytochemistry have shown that human tumors from the breast (Bartek *et al.*, 1990), liver (Bressac *et al.*, 1990), lung (Iggo *et al.*, 1990; Takahashi *et al.*, 1989), colon (Nigro *et al.*, 1989; Rodrigues *et al.*, 1990), and soft tissue (Mulligan *et al.*, 1990) commonly contain deletion and/or mutation of the p53 gene. In addition, there are reports of human tumors lacking both copies of p53 (Mulligan *et al.*, 1990) and therefore producing no p53 protein. The accumulated data

indicate that genetic alterations in the p53 gene are frequently involved in human oncogenesis.

It is becoming clear that both expression of mutant p53 protein, and lack of expression of the normal p53 protein can contribute to carcinogenesis. For example, Finlay *et al.*, (1988), Halevy *et al.*, (1990) and Baker *et al.*, (1990) reported that specific mutations in the p53 gene cause cells to produce mutated proteins that did not transform normal cells. In contrast Eliyahu *et al.*, (1988) reported that some mutations may be dominantly acting in that they inhibit the function of normal p53 protein by producing protein that complexes with this protein. The work of Baker *et al.*, (1989) supports the concept of dominant acting p53 proteins, in that some human colon tumor cell lines which carry a mutant p53 protein have also lost the other copy of the p53 gene. Tumorigenicity in this case is probably due to the dominant acting mutated p53 protein as well as loss of the suppressive function of the normal p53 gene. It is clear from these studies that expression of a mutant p53 protein as well as absence of the normal p53 protein can contribute to human carcinogenesis.

Wilms' Tumor Gene

Wilms' tumor, otherwise called nephroblastoma, occurs in the kidney of young children. Tumors can occur bilaterally or unilaterally and are present with other urogenital abnormalities (Matsunaga, 1981). Riccardi *et al.*, (1980) and Francke *et al.*,

(1979) reported that constitutional deletions in chromosome 11 were associated with urogenital abnormalities. Knudson (1971) reported that this deletion is associated with bilateral Wilms' tumor. Recently, the Wilms' tumor I gene has been isolated and is located at 11p13 (Grundy *et al.*, 1988).

DCC Gene

Frequent losses on the long arm of chromosome 18 around 18q21 have been reported in colorectal carcinomas but not adenomas (Vogelstein *et al.*, 1988). This site contains a gene termed DCC for "deletion in colon carcinoma" which could be a tumor suppressor gene (Volgelstein *et al.*, 1988). It has not yet been cloned.

Evidence for Suppressor Gene Inactivation In Chemical Carcinogenesis

There is a growing body of evidence to suggest that loss of suppressor gene function plays a significant role in the tumorigenic process. For example, benzo[a]pyrene-transformed tumorigenic Syrian hamster cells fused with non-tumorigenic immortal cells resulted in hybrids that lost the anchorage independent and the tumorigenic phenotype (Koi and Barrett, 1986). From this study the authors concluded that there was a loss of tumor suppressor gene function in the progression of benzo[a]pyrene induced transformation and tumorigenicity of this Syrian hamster cell line. They also argued that in chemical carcinogenesis, neoplastic progression requires oncogene activation, induction of immortality/or an extended life span and loss of tumor suppressor gene function. Evidence to support this hypothesis was reported in a study by Ruggeri *et al.*, (1991) who showed that in the two stage skin carcinogenesis

protocol, (discussed earlier) 25-50% of the carcinogen induced murine skin tumors had genetic alterations in the p53 gene, a well characterized tumor suppressor gene. These alterations included G->C transversion mutations and loss of heterozygosity. These genetic alterations were associated with tumors possessing a poorly differentiated and/or a highly malignant phenotype. The findings by Yang *et al.*, (1992) provided additional evidence that loss of the genetic material contributes significantly to neoplastic progression in chemical carcinogenesis. They reported that BPDE-transformed an infinite life span, human fibroblast (MSU-1.1) to fibroblasts that induced benign as well as malignant tumors. The high grade malignant tumors had greater loss of genetic material than the low grade malignant or benign tumors. Collectively, these data strongly indicate that the genetic change in this case could involve loss of tumor suppressor gene function.

IN VIVO STUDIES

Studies in carcinogenesis have utilized animal models to assess the development of cancer as it relates to humans. These studies provide important data on biotransformation and metabolism of some compounds that are known to be carcinogenic. The major objective of most studies is to analyze carcinogenesis in a specific organ. For example, the rodent model is widely used to study cancer development in the liver, kidney, brain and mammary gland (cf., Farber, 1982). Two of these systems will be briefly discussed.

Rodent Hepatic Carcinogenesis

Chemical carcinogenicity studies using rodent systems have provided significant information on chemicals with carcinogenic potential to humans. Earlier work in rat hepatic carcinogenesis utilized dietary administration of carcinogens for the life span of the animals. When agents like diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) were used, foci, nodules, adenomas and hepatocellular carcinomas appeared in rapid succession and most animals eventually died from hepatocellular carcinomas after six months (Pitot *et al.*, 1978; Kitagawa and Sugano, 1978; Williams *et al.*, 1981; Schulte-Hermann *et al.*, 1982). The development of lesions in a sequential manner suggests a multi-step process in this model of hepatic carcinogenesis. This animal model has certain limitations since: (1) the foci observed could have been initiated at different times due to the continual presence of the carcinogen which might obscure the step-wise nature of the process, (2) these compounds induce necrosis of hepatocytes, the concomitant proliferation of parenchymal as well as non-parenchymal cells can often times complicate both the histological as well as the biochemical parameters, (3) the prolonged presence of a carcinogen causes necrosis and some degree of reversible and regenerative hyperplastic lesions that are not related to the carcinogen (cf., Gunnar and Seglen, 1990).

The test results from animal models of this type that are used to predict human risk are often complicated by other biological factors including the species and strain of animal used. For example, B6C3F1 mice develop spontaneous hepatic tumors at an

incidence of 20-30%. The high incidence of tumor development in this strain calls into question the significance and the reliability of using data from such animals to predict human risk (Fox *et al.*, 1990).

In order to understand and define the events that occur at different times in chemical carcinogenesis, recent studies have focused on initiation-promotion protocols. Several types of protocols exist (Solt and Farber, 1976; Goldsworthy *et al.*, 1986; Pitot *et al.*, 1988) but one of the most widely used is the Solt-Farber technique described in 1976. In this model, rats are administered a high dose of carcinogen (e.g., DEN) to act as an initiator. Initiated cells are selected by a combination of feeding a low subcarcinogenic dose of 2-AAF for a short period followed by surgical partial hepatectomy (PH). 2-AAF is mitoinhibitory to normal liver cells whereas initiated cells continue to proliferate and PH acts as a mitogenic stimulus for initiated cells. This popular model has the advantage of rapidly inducing pre-neoplastic lesions which rapidly progress as a cohort into nodules, adenomas and finally to carcinomas approximately one year after the initial treatment. This model also makes it possible to study the genetic events that cause progression from foci-> hepatic nodules-> adenomas and finally to carcinomas.

In such studies, administration of an initiating agent alone does not cause tumor formation (cf., Pitot and Dragan, 1991). After the initiating agent, a tumor promoting compound is administered for an extended period. Promoting agents like phenobarbital and related compounds have been shown to induce the development of

pre-neoplastic foci to neoplastic nodules, adenomas and hepatocellular carcinomas (Pitot *et al.*, 1978; Scherer, 1984; Peraino *et al.*, 1988). The promoting agent primarily induces increased cellular proliferation which causes the appearance of clonal proliferations known as foci. Some of these foci persist following cessation of promoter treatment but others regress. Promoting agents are usually administered at noncytotoxic doses and are devoid of detectable mutagenic activity.

Some tumor promoters cause growth of hepatocytes, others cause induction of hepatocellular drug metabolizing enzymes still others cause proliferation of peroxisomes. In some cases, administration of non-initiating compounds which cause peroxisome proliferation leads to the development of neoplastic nodules and finally to hepatocellular carcinomas after 1-2 years (Rao *et al.*, 1984a; 1987). Some peroxisome proliferators induce foci and nodules that show a spectrum of enzymatic changes that are unique and have not been reported to be associated with other hepatic carcinogens (cf., Gunnar and Seglen, 1990). The ability of peroxisome proliferators to act as complete carcinogens could be related to the elevated intracellular levels of hydrogen peroxide produced by peroxisomal beta-oxidation of fatty acids, which in turn leads to the formation of free radical oxygen species which cause DNA damage (Rao *et al.*, 1984b; Conway *et al.*, 1987). This suggestion is supported by the fact that peroxisome proliferators show no direct mutagenicity in short term in vitro assays (Rao *et al.*, 1987; Conway *et al.*, 1987; Cattley *et al.*, 1986).

Studies have indicated that the effects of some initiating agents can be prevented with the use of antioxidants (Sato *et al.*, 1984; Cerutti, 1985; Williams *et al.*, 1986; Ito and Hirose, 1987). These antioxidants are postulated to inhibit hepatic carcinogenesis through: (1) induction of a number of carcinogen-detoxifying enzymes, (2) inhibition of oxygen free radical formation, or, (3) antiproliferative effects through the inhibition of ornithine carboxylase an important enzyme in polyamine synthesis (Sato *et al.*, 1984; Williams *et al.*, 1986; Ito and Hirose, (1987).

The effects of antioxidants vary according to the type of protocol and initiating agents used. For example, Williams *et al.*, (1986) reported a dose-related decrease in the number of altered foci and hepatocellular carcinomas in animals fed butylated-hydroxyanisole or butylated-hydroxytoluene with long term administration aflatoxin B1. However, in the same study prolonged administration of antioxidants after termination of carcinogen treatment failed to reduce the incidence of hepatocellular carcinomas. Other studies by Moore *et al.*, (1986) and Thamavit *et al.*, (1985) reported that antioxidants caused inhibition of foci formation during the early initiation/promotion phase of short term protocols. These data indicate that antioxidants act at the early initiating step but not at the later stages, providing indirect evidence to support the multi-stage nature of carcinogenesis.

Molecular analysis of chemical carcinogen induced hepatic rodent tumors indicate that alteration in expression and/or mutations in a number of important cellular genes like *ras, myc, raf and fos* are likely to be playing an important role in the development

of hepatic carcinogenesis (cf., Gunnar and Seglen, 1990). Vesselinovitch and Mihailovich (1983) suggested that at least four independent events are required for liver carcinoma formation. Collectively, these data indicate that the development of hepatic neoplasia involves multiple genes and or/events and supports the multi-stage concept of carcinogenesis.

Mammary Carcinogenesis

Another model system for tumorigenicity *in vivo* utilizes rats and mice to study chemically induced mammary neoplasia (Gullino *et al.*, 1975; cf Guerrero and Pellicer, 1987). Early studies by Huggins *et al.* (1961) showed that a single dose of methylcholanthrene or dimethylbenz[a] anthracene (DMBA) in sesame oil at 50-65 days of age caused mammary tumors in 100% of female Sprague-Dawley rats. In contrast, administration of the carcinogen at an earlier or later age caused a much lower incidence of tumors. Huggins postulated that the hormonal status of the animal could greatly influence the development of mammary tumors. Using this model, Sukumar *et al.* (1983) reported that the carcinogen NMU, induced carcinomas of the mammary gland in the rat. Many other carcinogens have been used to cause tumors in this model. Since the tumors typically develop after a long latency (6-12 months) it suggests that additional changes are required for tumor development. In this case hormonal stimuli act as promoters and facilitate these changes since ovariectomy of rats before carcinogen treatment markedly decreases the incidence of tumors (Gullimo *et al.*, 1975). If the animals were ovariectomized after carcinogen treatment rather

than before, 90% of the animals developed tumors. However these tumors developed after a very long latency and the average number of tumors per animal decreased. It seems clear in this model that formation of tumors in the mammary gland of the rat, after exposure to a carcinogen, e.g., NMU, is an example of the initiation-promotion procedure described earlier, if one considers the carcinogen to be the initiating agent and hormones to be the promoter.

More recently, the identification of activated proto-oncogenes in chemically-induced tumors has prompted investigators to try to identify specific genes that are activated in mammary tumors as a result of exposure to chemical carcinogens. For example, Kumar *et al.* (1990) reported that the *ras* proto-oncogene was activated during the initiation stage of rat and mouse mammary carcinogenesis but additional genetic changes were necessary for the development of malignant tumors. These data provide further convincing evidence for the multi-step nature of the carcinogenic process.

Human Colon Cancer

The development of human cancer involves multiple steps and several types exhibit these characteristics. One of the best characterized is colorectal cancer. Tumors of the colon and rectum represent one of the most common neoplasms in humans (Foulds, 1958). Most of these tumors are benign epithelial polyps (adenomas) with an incidence of 25-50% in older adults (Chapman, 1963). These adenomas are important because they represent precursors to colon cancer (Feneogline and Lane, 1974; Sugarbaker *et al.*, 1985). The progression from benign polyps to malignant colorectal

carcinomas provides an excellent model to study the "step-wise" genetic alterations involved in the development of this type of human cancer. Most colon cancers appear sporadically in which case it is assumed that factors such as diet play an important role (Fearon and Vogelstein, 1990). However, a small percentage of patients with colon cancer inherit an autosomal dominant syndrome, familial adenomatous polyposis (FAP), in which hundreds of colorectal adenomas develop in affected persons (Haggit and Reid, 1986). Unless the colon is removed, 100% of FAP patients will develop malignant colorectal tumors by age thirty (Robbins, Kumar and Cotran 1989). Recently, a genetic defect in this disease was identified. A gene named *fap* which was mapped to chromosome 5q (Bodmer *et al.*, 1987, Lipkin, 1988) is hypothesized to be directly involved in epithelial hyperproliferation that precedes the onset of polyps, adenomas and carcinomas in the colon. Vogelstein *et al.*, (1988) proposes that the *fap* gene normally functions as a negative regulator of colonic epithelial proliferation. When one copy of the gene is inactivated by mutation or deletion, the resulting decrease in normal gene expression is insufficient to control proliferation even when the other wild-type allele is functional. Changes in the *fap* gene creates a selective growth advantage in these cells so that by clonal expansion numerous progeny of transformed cells with this change arise. This increases the chances that additional changes related to carcinogenesis will occur in a cell that has already acquired the previous change (Fearon and Vogelstein, 1990). Evidence for this hypothesis comes from a study by Fearon *et al.*, (1987) who showed that the very small adenomas were

comprised of a monoclonal population of cells unlike the colonic epithelium which is polyclonal. Ponder and Wilkinson (1986) reported that adenomas arise from a single epithelial stem cell which is consistent with the idea that a single cell within the epithelial pocket initiated the neoplastic process and through clonal expansion formed a benign tumor (polyp). The development of malignant tumors required additional changes and the molecular alterations identified accumulated in a fashion that paralleled the clinical progression of the tumors (Vogelstein *et al.*, 1988). The fact that many genetic changes are required for tumor formation presumably explains why it takes years for progression from an adenoma to a carcinoma. Using colon tumors from FAP patients, as well as non-FAP patients Vogelstein *et al.*, (1988) systematically examined the genetic alterations that occur during colorectal development. They concluded that mutational activation of oncogenes coupled with the loss of expresssion of several other genes (that normally suppress tumor growth) are required for colorectal tumorigenesis.

One of the most important types of somatic alteration identified in colorectal tumors is *ras* gene mutation. Bos *et al.*, (1987) and Forrester *et al.*, (1987) reported that 50% of colorectal carcinomas had a *ras* gene mutation. Adenomas greater than 1 cm in diameter have a similar frequency of mutation in the *ras* gene (Vogelstein *et al.*, 1988). In contrast, tumors less than 1 cm in diameter have a much lower incidence of *ras* mutations and such tumors rarely progressed to malignant carcinomas. It is not clear

whether the *ras* mutation is the initiating event or whether it is responsible for the progression from adenoma to carcinoma.

Evidence that inactivation of tumor suppressor genes contributes to colorectal carcinogenesis is the fact that 75% of the tumors examined show a deletion on chromosome 17p and 18q (Vogelstein *et al.*, 1988; Delattre *et al.*, 1989). These two chromosomes contain putative suppressor genes. In the case of chromosome 17, Baker *et al.*, (1989) identified the common region lost as 17p, the region which contains the p53 gene. He also noted that in some cases, colorectal tumor cells have mutations in the p53 gene, these cells also lack the normal allele thereby producing only the altered form of the gene product. A genetic defect involving chromosome 18q has been mapped. The putative tumor suppressor gene at this region has been termed DCC (Fearon *et al.*, 1990). From the studies described it is clear that at least four potential genetic alterations are required for colorectal tumor formation. These are, *ras* gene mutations and point mutations and/or deletions of specific genes located on chromosome arms 5q, 17p and 18q. These genetic alterations may occur in a preferred sequence but it is the total accumulation of changes, rather than their order that is responsible for determining the tumor's biological properties.

IN VITRO STUDIES

Neoplastic transformation *in vitro* is an acceptable model of tumor formation *in vivo*. The demonstration that cells acquire independent phenotypic changes such as

morphologic transformation, anchorage independence and growth in medium without exogenous growth factors, makes clear the multi-step nature of the process (McCormick and Maher, 1988, 1990). Some of the results of transformation studies using oncogene transfection or carcinogen tratment of fibroblasts in vitro will be discussed.

Rodent Fibroblast Cell System

The initial studies in *in vitro* cellular transformation utilized rodent fibroblasts such as Syrian hamster embryo (SHE) cells which are diploid and have a finite life span. Well-characterized mouse cell lines such as C3H10T1/2 fibroblasts and BalbC/3T3 fibroblasts have also been used. The use of rodent cells for oncogene transfection and/or treatment with carcinogens have provided valuable information on the mechanisms of carcinogenesis. For example the discovery of human oncogenes by Weinberg and his colleagues (Shih *et al.*, 1981) was accomplished using the infinite life span mouse fibroblast cell line NIH3T3. Such studies with rodent fibroblasts provided a strong background for similar studies using human fibroblasts.

Oncogene Mediated Transformation Of Rodent Fibroblasts

One of the major advances in recent years, was the demonstration in 1982 that the transforming genes responsible for some human tumors, were the homologue of the *v-ras* gene which plays a causal role in rodent sarcomas. These transforming human genes could be detected by transfection of human tumor cell DNA samples into NIH3T3 cells (Parada *et al.*, 1982; Der and Cooper, 1983). Since the *ras* and *myc*

genes are among the best characterized this brief discussion will focus on these two genes.

Simultaneous transfection of finite life span rat fibroblasts with the *ras* and *myc* oncogenes induced cellular transformation *in vitro* and the cloned progeny formed tumors when injected into athymic mice (Yagi *et al.*, 1989). Transformation of these cells required the expression of both genes at higher levels than normal. They also reported that "early stage" fetal cells exhibited a higher frequency of transformation than "late stage" more differentiated cells which exhibited a lower frequency of transformation. The authors suggest that additional genetic events, in addition to the overexpression of the two transfected oncogenes, were responsible for malignant transformation of finite life span diploid rat fibroblasts. Other reports also indicate that transformation of normal rodent fibroblasts *in vitro* requires at least two cooperating oncogenes (Land *et al.*, 1983; Katz and Carter, 1986).

Egan *et al.*, (1987) found that transfection of the immortalized mouse fibroblast cell line (C3H10T1/2) with the H-*ras* oncogene caused the cells to be morphologically transformed. These transformed cells induced tumors in athymic mice. The degree of morphologic transformation of the cells in culture correlated positively with the level of *ras* mRNA expression. In similar studies Pulciani *et al.*, (1982) and Spandidos and Wilkie (1984) reported that NIH3T3 cells can be transformed to malignancy by transfection of these cells with the H-*ras* oncogene designed to be expressed at high levels. NIH3T3 cells are immortal and have other undefined genetic alterations which

are thought to complement the *ras* oncogene in causing malignant transformation. Another type of study that supports the transforming potential of the H-*ras* oncogene in mouse fibroblasts was reported by Stacey and King, (1984). Using NIH3T3 cells, they found that microinjection of the purified H-*ras* oncogene protein induced morphologic transformation and an increased rate of ^{3}H uptake compared to the control cells injected with albumin. In a similar study Feraminisco *et al.*, (1985) showed that microinjection of antibodies to the H-*ras* oncogene product caused transient reversion of the transformed morphology.

The evidence for the transforming ability of the H-*ras* oncogene (mutated in codon 12 or 61) is clear. There are also a few reports that the H-*ras* proto-oncogene can be activated by upregulation. For example, Yancopoulos *et al.*, (1985) reported that high levels of expression of the H-*ras* proto-oncogene caused normal rat embryo fibroblasts to express some transformed properties. However, these transformed cells were not tumorigenic. In a similar study Ricketts and Levinson (1988) reported that high expression of the H-*ras* proto-oncogene caused "partial transformation" of rat-1 cells in culture. It appears that increased expression of the *ras* proto-oncogene by itself has limited transforming potential as compared to its mutated counterpart which has strong transforming capabilities even when the mutated gene is expressed at lower levels.

Transformation of Rodent Cells by Carcinogen Treatment

SHE cells have been used extensively in carcinogenesis studies because *in vitro* one can take these normal diploid cells and transform them to cells with tumorigenic properties (Barrett and Ts'o, 1978). Berwald and Sachs (1963) were the first to report carcinogen-induced cellular transformation *in vitro* of Syrian hamster embryo fibroblasts. In that report they observed that chemical carcinogens induced changes in morphology of SHE cells grown in culture. When these morphologically transformed cells were passaged many times and then transplanted into syngeneic animals, the transplanted cells induced tumors. However, when early passage transformed cells were transplanted, the cells did not induce tumors. From many later studies, it is apparent that by passaging of the cells in culture for extended periods, the cells acquired additional genetic changes, which collectively, resulted in the expression of the tumor phenotype. In 1978, Barrett and Ts'o carried out a similar experiment treating SHE cells with benzo[a]pyrene. The treated SHE cells formed foci. The cloned, focus derived, progeny were morphologically transformed and after extended sub culturing these cells were anchorage independent and expressed enhanced fibrinolytic activity. These phenotypes were obviously acquired independently of each other indicating that separate genetic events (steps) were required for tumorigenicity. Recent studies have tried to define these steps. In 1982, Newbold *et al.*, and later Koi and Barrett (1986) suggested that chemically induced neoplastic transformation of SHE cells involves at least three steps. These are induction of immortality, proto-

oncogene activation and/or loss of tumor suppressor gene function. More recently Ebert *et al.*, (1990) reported that treatment of SHE cells with diethylstilbestrol (DES) induced a significant elevation in H-*ras* expression whereas benzo[a]pyrene and trenbolone caused enhanced expression of the c-*myc* gene. Collectively these studies, using hamster fibroblasts, provide evidence that cellular transformation *in vitro* is the result of a multistep process.

Another approach for studying carcinogenesis *in vitro* utilizes well-established, immortal cell lines such as C3H10T1/2 and Balb 3T3 cells. Extensive transformation studies on C3H10T1/2 cells have been carried out using chemical carcinogens as well as ionizing radiation. For example, in studying the relationship between x-ray exposure and malignant transformation in C3H10T1/2 cells Kennedy *et al.*, (1980a) reported that few if any of the transformed clones occurred as a direct result of the x-ray exposure. The reason for this conclusion was that the number of transformed foci per dish was independent of the number of irradiated cells placed in a dish. They hypothesized that in this case, malignant transformation required at least two steps. Exposure to x-ray induced one change which occurred in all the irradiated cells and was transmitted to all the progeny cells. This change enhanced the probability of a second spontaneous event leading to malignant transformation. Malignant transformation, however, still required extensive subculturing of the cloned progeny.

In other studies Kennedy *et al.*, (1978), Kennedy and Little (1980b), Miller, *et al.*, (1981), Han and Elkind, (1982) have reported that repeated treatment with the tumor

promoter TPA, after x-ray treatment of these cells, markedly increases the frequency of transformed foci. These data as well as the studies reviewed by Landolph (1985) and Herschman and Brankow (1986) show that transformation requires multiple changes and immortal cell strains such as C3H10T1/2, have already acquired one or more of these changes.

Human Fibroblast Cell System

While studies on rodent fibroblasts *in vitro* provided most of the early conceptual framework on carcinogenesis the ultimate goal of most cancer research is to understand how the disease originates in humans. Epidemiological studies indicate that human cancer can develop from the exposure to carcinogens in the environment (Doll, 1980) but *in vitro* spontaneous malignant transformation of normal human cells in culture or after carcinogen treatment has never been observed. (cf., McCormick and Maher, 1988; Chang, 1986). Recently however, a number of workers using different strategies have successfully transformed normal human fibroblasts to tumor cells. Some of these results will be discussed briefly.

Oncogene Mediated Transformation of Human Fibroblasts

With the discovery that oncogenes exist in tumors (Shih *et al.*, 1981) researchers hypothesized that transfection of normal, diploid, human cells such as fibroblasts with appropriate oncogenes should also make these cells tumorigenic. Early studies however, met with limited success. Sager *et al.*, (1983) reported that finite life span diploid human fibroblasts were resistant to malignant transformation when they were

transfected with the *ras* oncogene, a cloned transforming gene from a human tumor. Similar findings were reported by Hurlin *et al.*, (1987) and Wilson *et al.*, (1989) using finite life span diploid human fibroblasts transfected with vectors designed for high expression of the H- and N-*ras* oncogenes, respectively. They reported that high expression of the H-or N-*ras* oncogene induced morphological transformation and the ability to make small clones in soft agarose, but the transformed cells did not acquire an infinite life span and did not form malignant tumors in athymic mice.

Several workers recognized that in order for normal human fibroblasts to be transformed to malignant fibroblasts in culture, the fibroblasts to be transformed must acquire a greatly extended and/or an infinite life span. For example, using SV40 immortalized human fibroblasts (Sack, 1981) Sager and her colleagues (O'Brien *et al.*, 1986) compared the tumorigenic capabilities of immortal and non-immortal cells. They found that non-immortal fibroblasts infected with the Harvey or Kristen murine sarcoma virus, which carry *ras* oncogenes, did not form tumors when the virus infected cells were injected subcutaneously into an appropriate host. However, when the infinite life span cells were infected with the same virus the infected cells acquired some transformed phenotypes and induced "nodules" when transplanted into athymic mice. Some of these nodules regressed, others remained static. Since histopathology was not reported, the exact nature of these growths is unclear. Those studies as well as others (Suarez *et al.*, 1987; Namba *et al.*, 1986; McCormick and Maher, 1988) suggest that acquisition of an infinite life span is an essential pre-requisite for

malignant transformation of human fibroblasts *in vitro*. After reviewing all published studies claiming to neoplastically transform human fibroblasts in culture McCormick and Maher (1988) came to the conclusion that no one had successfully transformed human fibroblasts in culture to tumorigenic cells. They suggested that for such studies to succeed, one needed to have immortal human fibroblasts. For mechanistic studies, it was preferable that these cells would be diploid, non-tumorigenic and have no transformed characteristics. Such a cell strain was developed by transfection of the v-*myc* oncogene and designated MSU-1.0 (Morgan *et al.*, 1991). Initially, these cells grew very slowly but after several months these cells began replicating more rapidly. These rapidly proliferating cells were identified as variants and were called MSU-1.1. Karyotypic analysis showed that these cells were clonally-derived since they had 45 chromosomes including two marker chromosomes. Southern blot analysis showed that both cell strains had the same integration site for the v-*myc* oncogene and both expressed the v-*myc* oncoprotein. These data suggest that MSU-1.1 was derived from MSU-1.0. The MSU-1.0 cells cannot grow without exogenously added growth factors. However the MSU-1.1 cells grow moderately well under the same conditions and reach a higher saturation density than MSU-1.0 cells. The results of experiments using the MSU-1.1 cell strain indicate that H-, (Hurlin *et al.*, 1989), N-, (Wilson *et al.*, 1990), and v-Ki-*ras* oncogenes (Fry *et al.*, 1990) transformed MSU-1.1 cells. The transformed progeny cells induce rapidly growing and progressively invasive sarcomas when injected into athymic mice. The logical interpretation of these studies is that the

infinite life span phenotype of MSU-1.1 cells complemented the expression of the *ras* oncogenes in malignant transformation. Using a similar approach, Kinesella *et al.*, (1990) came to the same conclusion and reported that immortalization was necessary for the induction of malignant tumors using human fibroblasts infected with the N-*ras* oncogene.

Transformation of Human Fibroblasts by Carcinogen Treatment

It is now well-recognized that malignant transformation involves multiple genetic changes within a cell. For this to occur as a result of carcinogen treatment, requires successive clonal selection of cells containing each change. One explanation for the earlier failure to induce malignant transformation of human cells *in vitro* by carcinogen treatment could be the inability of researchers to recognize cells that have acquired intermediate changes (phenotypes) which would allow them to isolate these cells, expand the population, expose the cells a second time so as to cause further changes and eventually identify a malignant cell. The approach used by Namba *et al.*, (1978, 1981, 1985) was to repeatedly treat diploid human fibroblasts with ionizing radiation or the chemical carcinogen 4-nitroquinolone (4 NQO) with the hope of developing a transformed cell. This method did not involve any specific selection technique. However, they were successful in developing two immortal fibroblast cell strains KMST-6 and SUSM-1. These cells though not tumorigenic, form small colonies in soft agarose (i.e., anchorage independent), exhibit altered morphology, have a reduced requirement for exogenous growth factors and are chromosomally abnormal. Namba

and colleagues (Namba *et al.*, 1986, 1988) later found that when the immortalized human fibroblast cell strain, KMST-6 was infected with the Harvey sarcoma virus (Namba *et al.*, 1986) or transfected with an activated *ras* gene in a viral-like construct (Namba *et al.*, 1988) the *ras* oncoprotein caused the KMST-6 cells to have properties similar to tumor cells. The transformed cells gave rise to progressively-growing invasive fibroblastic tumors in athymic mice.

A typical example of recent success in malignant transformation using carcinogens, is the treatment of an infinite life span, human fibroblast cell strain MSU-1.1 with a reactive derivative of the carcinogen benzo(a) pyrene refered to as BPDE (Yang *et al.*, 1992). They reported that a single treatment with BPDE induced foci formation. Selected cells from these foci were found to exhibit anchorage independent growth and acquired the ability to grow in medium without exogenous growth factors. The cloned, focus-derived, progeny induced benign and malignant tumors when transplanted into athymic mice. The induction of morphologically-distinct tumors clearly demonstrates that different genetic events can cause neoplastic transformation, and these data provide further evidence for the multi-stage nature of carcinogenesis. In another study, (Yang, 1992) the infinite life span, diploid, parental cell strain of MSU-1.1 called MSU-1.0, failed to be transformed to full malignancy after repeated treatments with the same carcinogen after progressively more stringent selection of transformed clones. Some transformants from these experiments exhibited morphological transformation, anchorage independent growth, grew in medium

without exogenous growth factors and formed poorly defined, subcutaneous nodules in athymic mice that regressed after a short period. In no instances did any of the cloned progeny cells give rise to unequivocal malignant tumors. This study is interpreted to indicate that the more "normal" the cell the greater the number of independent steps required for the development of the malignant phenotype.

In summary, the results of studies discussed here indicate that human fibroblasts are not refractory to malignant transformation. By repeated clonal selection to yield a population of cells that express the appropriate phenotypic changes, tumorigenic malignant cells can be isolated.

RAS GENE FAMILY

To date more than 60 different oncogenes have been identified. However, the *ras* gene is the most studied and best characterized (cf Shih and Weeks, 1984; Barbacid, 1987). The H- and K-*ras* genes were identified as the transforming agents in the induction of sarcomas by the Harvey and Kirsten strains of murine sarcoma viruses (Ellis *et al.*, 1986; Tsuchida *et al.*, 1982; cf Spandidos and Lang, 1989). These murine viruses captured the *ras* gene from normal cells in the infection process. When these genes (*ras*) were transfected into murine fibroblasts such as NIH 3T3, the fibroblasts were transformed, indicating that they are potential transforming genes (cf Barbacid, 1987).

The human *ras* gene family consists of a group of highly conserved sequences designated H-*ras*-1, (Chang *et al.*, 1982a), K-*ras*-2 (MacGrath *et al.*, 1983) and N-*ras*

(Hall *et al.*, 1983). The H-*ras* gene is located on chromosome 11, the K-*ras* gene is located on chromosome 12 and the N-*ras* gene is located on chromosome 1. The three genes have a similar structure in that they are comprised of 4 exons separated by 3 introns and the genes code for a 21kD protein (p21) made up of 189 amino acids (Taparowsky *et al.*, 1983). The K-*ras* gene is unique in that it has two fourth exons which result in two distinct *ras* proteins, each with a different structure and function (McGrath *et al.*, 1983). It also has a fifth exon designated exon 0 which is located upstream from the 4th exon. The function of the 0 exon and of two alternative fourth exons are not known. Cichutek and Duesberg (1986) reported that the two alternative fourth exons may be involved in translation of *ras* mRNA and if so, changes within these non-coding sequences might alter the level of protein expression.

Mechanism of Cellular Activation of *Ras* Genes

The two broad categories of activation of the *ras* genes are changes in quantitative expression and changes in qualitative expression (Barbacid, 1987). Changes in quantitative expression of the *ras* proto-oncogene can occur in some cases where the gene is not mutated. For example, in instances where the *ras* gene is linked to the retroviral transcriptional enhancer, this linkage causes marked increases in expression of the *ras* gene which in turn confers the transformed phenotype to cells (DeFeo *et al.*, 1981; Pulciani *et al.*, 1985). The work of Ricketts and Levinson (1988) indicates that increased expression of the *ras* proto-oncogene has limited transforming potential. However, increased expression of the *ras* gene has been reported in a number of

tumors. For example, some chemical carcinogen-induced mouse skin tumors (Quintinilla *et al.*, 1986; Bizbud *et al.*, 1986) and liver tumors (cf Gurrero and Pellicer, 1987) had a variable frequency of increased expression of the H-*ras* proto-oncogene. There are also several reports of increased expression of the *ras* gene product in human tumors. Noguchi *et al.*, (1986) found that parietal cells of the gastric epithelium as well as gastric adenocarcinomas expressed increased amounts of *ras* p21 protein. In a similar study Rjinders *et al.*, (1985) and Viola *et al.*, (1986) showed that over 50% of prostatic carcinomas and bladder carcinomas had an increased expression of *ras* p21 protein while benign, proliferative, or normal tissues from either organ did not show increased levels of *ras* protein expression. Similar results have been found by Hand *et al.*, (1984), Spandidos and Kerr (1984) and Gallick *et al.*, (1985) in colon carcinomas. They found an increased expression of *ras* p21 protein in some carcinomas and adenomas, but the normal colonic epithelium did not show elevated levels of *ras* expression. One study (Feinberg and Vogelstein, 1983) reported that the *ras* gene was hypomethylated in six of eight (75%) primary colorectal tumors examined, and this hypomethylation correlated with increased expression of the gene. It has been postulated that this hypomethylation might play a role in the increase in p21 *ras* expression observed in these tumors.

Qualitative changes in *ras* expression usually result from point mutations in the coding sequences of the *ras* gene (Patterson *et al.*, 1987; Reynolds *et al.*, 1987; Feraminsco *et al.*, 1985; Wilson *et al.*, 1990; Hurlin *et al*, 1989). The point mutations

cause a single base pair change in the cellular DNA sequence resulting in a change in the amino acid composition of the protein molecule. Point mutations in the *ras* gene have been found in a wide range of human tumors (Tabin *et al.*, 1982; Reddy *et al.*, 1982; Santos *et al.*, 1983; Capon *et al.*, 1983). The transforming potential of specific mutations in the *ras* proto-oncogene have been well documented (Ferminisco *et al.*, 1985; Hurlin *et al*, 1987 and 1989; Wilson *et al.*, 1989 and 1990). *In vivo* studies also indicate that chemical carcinogen induced tumors frequently have activated *ras* genes (Newcomb *et al.* 1989; Bremner and Balmain, 1990; Brooks, 1989; Ebert *et al.*, 1990; Buchmann *et al.*, 1991). Additionally, Cohen and Levinson (1988) reported that a mutation in the fourth intron of the T_{24} H-*ras* oncogene causes a tenfold increase in p21 protein expression. However, point mutations in codons 12,13, 59 and 61 seem to be the sites most frequently involved in activation of the *ras* proto-oncogenes (Spandidos, 1989).

Ras Gene Product (p21) in Cell Proliferation

The *ras* proteins belong to a family of related polypeptides that are present in all eukaryotic organisms from yeast to humans. *Ras* proteins are a subclass of the G proteins that are involved in transmembrane signaling through generation of second messengers. Mature *ras* proteins are synthesized in the cytosol and become associated with the inner side of the plasma membrane after post-translational modifications. Their biochemical properties include binding and exchange of guanine nucleotides. The current model of *ras* function predicts that binding of external stimuli, e.g.,

growth factors like PDGF (Satoh *et al.*, 1990a) or EGF (Satoh *et al.*, 1990b) increases the formation of the active complex (p21GTP). This activated molecule undergoes conformational change and is able to interact with a cellular target(s). Normal and transforming *ras* proteins bind GTP with similar affinities, but the intrinsic GTPase activity of the transforming protein is impaired (cf Grand and Owen, 1991).

Santos and Nelereda, (1989) proposed a model in which the Gap protein molecule facilitates both interaction of *ras*GTP complex with the downstream target and subsequent deactivation to the *ras*GDP form. Oncogenic mutations cause *ras* proteins to stay preferentially in the active conformation and produce a continuous flow of signals resulting in cellular transformation.

The use of neutralizing antibodies has provided valuable clues as to the function of *ras* proteins. *Ras* transformed cells as well as tyrosine-kinase associated oncogenes such as *fms, src* or *fes* show transient reversion of the transformed morphology when injected with a *ras* monoclonal antibody (Santos and Nebreda, 1989). This effect was not seen when *ras* protein neutralizing antibody was injected into cells transfected with *Mos* and *raf-1* oncogenes which have serine or threonine kinase activity, respectively (Barbacid, 1987). These data indicate that the transducing proliferative signals of *ras* can originate from a variety of surface receptors and membrane associated molecules or their altered oncogenic versions. *Mos* and *raf* oncogenes are thought to act in totally separate pathways or in the same pathway downstream from *ras*. Alanso *et al.*, (1988) reported that cellular transformation induced by *mos* and *raf* produces changes

in the PI metabolism similar to those produced by transformation induced by membrane associated oncogenes. These data provide some support for the idea that these proteins act downstream from *ras*. Studies by Morrison *et al.*, (1988) support this hypothesis since they found that *ras* and membrane associated oncogenes, or cell surface receptor stimulation with growth factors, results in phosphorylation of *raf*-1 and activation of its serine/theroinine kinase activity. H-*ras*, oncogene mediated transformation results in activation of specific *jun*-like transcriptional factors which are also activated by phorbol esters and serum. Other oncogenes (membrane and cytoplasmic) can activate the same transcriptional factors (Santos and Nebreda, 1989). It is clear from these studies that *ras* acts as pivotal step in transmission of mitogenic signals from the surface receptors to nuclear transcriptional factors by integrating the different signals into a common pathway.

Ras Oncogenes in Animal and Human Tumors

Activated *ras* proto-oncogenes have not been found in non-tumor cells from patients whose tumor cells contain these oncogenes (Cline, 1989). This indicates that activated *ras* proto-oncogenes are the result of a somatic mutation and suggests they play a causal role in cancer development. Zarbl *et al.*, (1985) reported that female rats developed mammary carcinomas after a single dose treatment of MNU, a direct acting carcinogen. More than 80% of these tumors had a G-->A transition mutation in the H-*ras* proto-oncogene at codon 12. Similarly, Guerro *et al.*, (1984) reported that gamma radiation caused G-->A transition mutations in the K-*ras* proto-oncogene in

lymphocytes. Sukumar *et al.*, (1986) reported that methylnitrosamine induced mesenchymal tumors in the kidney and cells from these tumors had K-*ras* mutations. Similar mutations in the K-*ras* gene were also found in lung tumors induced by tetranitromethane (Stowers *et al.*, 1987). DMBA induced mouse skin tumors showed a specific A-->T transversion in codon 61 of the H-*ras* oncogene and this change has been found frequently in papillomas (Balmain and Pragnell, 1983; Bizub, *et al.*, 1986; Quintanilla *et al.*, 1986). Sinha *et al.*, (1988) found that aflatoxin induced liver tumors had mutations in the N-*ras* oncogene and similar mutations in the N-*ras* oncogene were also found when rat hepatocyte cell lines were transformed *in vitro* after treatment with aflatoxin.

Mutations in the human *ras* genes have been found in tumors of many different cell types and from different organs. Neri, *et al.*, (1988) found that the N-*ras* proto-oncogene was mutated in leukemic cells from patients with acute lymphoblastic leukemia. Activation of the *ras* gene in these tumor cells was caused by to G-->A transition mutation in codon 12 or 13.

The identification of *ras* oncogenes in human tumors as well as in spontaneous and chemically-induced animal tumors suggests that genes from this family play a causal role in the etiology of many types of cancer. This conclusion is supported by the high frequency in which mutations in *ras* genes are found in experimentally-induced tumors.

Ras Oncogenes In Metastasis

The cause of death in many human cancer patients is not the primary tumor but metastatic tumors that arise throughout the body. In order for tumor cells to metastasize several distinct steps must occur. First, tumor cells must detach from the "mass", then invade through a vessel wall, escape the immune system, survive in circulation, attach to a vessel wall, traverse the vessel wall again and finally proliferate in the distant organ (cf Nicolson, 1987). One of the first studies to indicate that, in tumor cells, expression of the *ras* oncoprotein may contribute to the cells metastasizing came from the work of Buckley (1985). He reported that high levels of expression of the *ras* oncoprotein caused detachment of *ras* transformed tumorigenic cells from each other and this correlated with an increased frequency of metastasis. The detachment of tumor cells from each other reflects changes in cell surface adhesion molecules particularly glycoproteins of which sialic acid is the major contributor. Roos (1984) and Mareel *et al.*, (1988) showed that an increase in the sialic acid residues decreases cell adhesiveness and enhances cellular invasion of chick hearts *in vitro* and metastasis *in vivo*. Bolscher *et al.*, (1986) showed that infection of mouse cells with the Kristen murine sarcoma virus and transfection of NIH 3T3 cells with the H-*ras* oncogene increased sialyation of membrane glycoproteins in these cells. These data provide evidence that expression of the *ras* gene product causes changes in some of the cell surface molecules that contribute to metastasis.

After tumor cells detach they must penetrate the extracellular matrix particularly the basement membranes. Recently, Jankun *et al.*, (1991) in our laboratory showed that *ras* transformed cells secreted high levels of plasminogen activators which can initiate a cascade of events leading to dissolution of the basement membrane and the extracellular matrix. Other evidence for this hypothesis came from Warburton *et al.*, (1986) and Isaacs *et al.*, (1988) who showed that transfection of *ras* oncogenes into tumor cells caused decreased fibronectin and collagen production both of which enhance metastasis. High levels of expression of the *ras* oncogene protein (p21) can induce mouse fibroblasts to increase production of proteases including collagenases, gelatinase, and cathepsin (Denhardt *et al.*, 1987; Alvarez and DeClerck, 1988) all of which can degrade matrix material. More significantly *ras* oncoproteins stimulate tumor cells to produce and secrete more type IV collagenase which destroys the basement membrane (Turpeenniemi-Hujanen *et al.*, 1984; Thorgeirsson *et al.*, 1985; Garbisa *et al.*, 1987). Liotta *et al.*, (1980) previously demonstrated a positive correlation between type IV collagenase and metastatic ability.

In circulation, tumor cells must escape the immune system if the metastatic process is to be completed. However, there are conflicting reports on the role of the *ras* genes on increasing the survival of tumor cells in circulation. Johnson *et al.*, (1985) and Trimble *et al.*, (1986) reported that transfection of the *ras* gene increases cell susceptibility to killing by natural killer cells (NK) *in vitro*. The reverse was found by

Thorgeirsson *et al.*, (1985) and Greenberg *et al.*, (1987) who reported that *ras* transformed cells did not show an increased susceptibility to killing by macrophages or NK cells. Hill *et al.*, (1988) reported that expression of the *ras* oncogene enhances the ability of cells injected intravenously to colonize the liver and lung. Altogether the evidence to support or confirm the role of *ras* oncogene in this phase of metastasis is unclear and requires further study.

Finally, at a distant site the cells must exit the blood or lymphatics vessels and proliferate. The *ras* oncogene plays a significant role in cell proliferation but exactly how this is accomplished in different mircoenvironments is not well understood (cf Nicolson, 1987). He also suggests that the ability of neoplastic cells to proliferate may reflect the failure of cells to form gap junctions with each other as well as with normal cells. In a later study, Nicolson (1988) reported that transfection of mammary adenocarcinoma cells with the *ras* oncogene caused inhibition of gap junction cell-to-cell communication and this inhibition correlates with an increase in the frequency of spontaneous metastasis by these cells. A decrease in gap junction and cell to cell communication of *ras* transformed malignant cells has been reported by Trosko *et al.*, (1990).

In summary these data provide some convincing evidence that the *ras* oncoprotein protein may mediate some of the biological events that are necessary for metastasis. It is also important to point out that other genes and/or molecular events can cooperate with the *ras* oncogene in inducing metastasis. Since, as noted, *ras* expression does not

consistently increase the frequency of metastatic lesions, the effect may be specific to certain cell types or cell lines.

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CHAPTER II

ROLE OF EXPRESSION LEVELS OF H-*ras* ONCOPROTEIN ON *IN VITRO* AND *IN VIVO* TRANSFORMATION OF AN INFINITE LIFE SPAN HUMAN FIBROBLAST CELL STRAIN MSU-1.1[1]

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[4]The abbreviations used are: DMSO, dimethylsulfoxide; FCS, fetal calf serum; McM, medium described in ref. 13; MT, metallothionein promoter; PBS, phosphate buffered saline; SCS, supplemented calf serum; SR_3 serum replacement supplements of ref. 14, but lacking epidermal growth factor and with the concentration of bovine serum albumin reduced to 0.25μg/ml.

ABSTRACT

When the near-diploid, infinite lifespan, non-tumorigenic, human fibroblast cell strain MSU-1.1 is transformed by a transfected H-*ras* oncogene expressed at high levels, the transformed cells exhibit many of the *in vitro* characteristics of tumor-derived cells and form malignant tumors with a very short latency. MSU-1.1 cells transformed by transfection of the same oncogene under the control of its endogenous promoter and expressed at the normal level do not do so. To analyze the role of H-*ras* oncogene expression in the malignant transformation of MSU-1.1 cells, we transfected them with a plasmid containing the metallothionein promoter fused to the T24 H-*ras* oncogene, and assayed the population for focus-formation in the presence or absence of zinc sulfate in the cell culture medium. Large-sized foci were found in populations given zinc, and a series of independent foci were isolated and assayed for zinc-induced changes in morphology. Five independent cell strains that exhibited significant changes in morphology in the presence of zinc and appeared morphologically normal in the absence of zinc, were chosen for further study. Each had a basal level of expression of *ras* p21 that was higher than the parental MSU-1.1 cells, and addition of zinc to the medium caused a dose-dependent increase in this expression. These MT-*ras* transformed cells formed tumors in athymic mice. Withdrawal of zinc supplemented drinking water had no effect on the growth pattern of tumors in tumor-bearing mice. Histologically, the malignant tumors were produced by transformed cells that expressed the H-*ras* oncoprotein at high levels and benign tumors were produced by transformed cells that expressed the H-*ras* oncoprotein

at low levels. These data indicate that high levels of the H-*ras* oncoprotein is required for malignant transformation of MSU-1.1 cells, a non-tumorigenic human fibroblast cell strain.

INTRODUCTION

Cell lines derived from human fibrosarcomas frequently exhibit activating mutations in *ras* proto-oncogenes. For example, the HT1080 and the SHAC fibrosarcoma cell lines, have a mutation in codon 61 of one copy of the N-*ras* gene (1, 2). Evidence to support the causal role of the N-*ras* oncogene in the transformation of HT1080 cells into tumorigenic cells comes from a study by Patterson *et al.* (4). They reported that a variant of HT1080 cells selected because they exhibited a flatter morphology in culture than the majority of such cells, no longer contained the chromosome carrying the mutant *ras* gene, and that the progeny of these cells no longer formed tumors. When a plasmid carrying the N-*ras* oncogene was transfected into these non-tumorigenic HT1080 cells and was expressed in them, the cells again became tumorigenic.

A number of other studies on mammalian cells in culture also support the hypothesis that *ras* genes can play a causal role in cell transformation. For example, it has been shown that mammalian cells in culture can be transformed into tumorigenic cells by significantly increasing the level of expression of *ras* proto-oncogenes, even without the proto-oncogene having an activating mutation (5-7). In other studies, McCormick and his colleagues showed that near-diploid, karyotypically stable, infinite life span human fibroblast cell strain they developed, designated MSU-1.1, can be transformed into malignant cells by transfection of an activated H-*ras* (8) or N-*ras* oncogene (9) expressed at a high level. However, if the MSU-1.1 cells express these transfected oncogenes at the normal level, neoplastic transformation does not occur.[3]

To demonstrate the effect of the level of expression of *ras* oncoprotein on the transformation of the human fibroblasts directly, we transfected MSU-1.1 cells with a plasmid containing the mouse metallothionein promoter fused to the coding region of the T24 H-*ras* oncogene derived from a human bladder carcinoma cell line. This is the H-*ras* oncogene that Hurlin *et al.* (8) used with a high expression vector to malignantly transform the MSU-1.1 cells. We then manipulated the level of H-*ras* p21 expression with zinc and examined the effect on the properties of the transfectants in culture and after subcutaneous injection into athymic mice. The plasmid used was that constructed and employed by Reynolds *et al.* (10) to examine the effect of H-*ras* oncoprotein expression on rat fibroblasts. Reynolds *et al.* found that rat cells in culture gave a morphological response and growth response that corresponded to the level of H-*ras* expression, but these investigators did not report any tumorigenicity studies. We chose the metallothionein promoter because studies by Reynolds *et al.* (10) indicated that it should be possible to administer zinc sulfate to human cells in culture at concentrations sufficient to induce H-*ras* expression. In mice, the normal level of zinc in the serum is low. Therefore, mice should tolerate an increase in exposure to zinc at levels that are sufficient to activate the metallothionein promoter as it did in transgenic mice (11, 12). The results of our study indicate that the level of expression of this H-*ras* oncogene controls the degree of *in vitro* transformation of the MSU-1.1 cells and their ability to form malignant tumors in athymic mice.

MATERIALS AND METHODS

Cells. Two human tumor-derived cell lines were used: T24 derived from a bladder carcinoma which has a H-*ras* oncogene expressed at high levels, and HT1080 derived from a fibrosarcoma which has an N-*ras* oncogene expressed at the normal level. These were obtained from the American Type Culture Collection. The MSU-1.1 cells were derived in this laboratory from a neonatal, foreskin-derived fibroblast cell line. Details of the development and characteristics of this cell line have been published (13). In brief, MSU-1.1 cells have a normal fibroblastic morphology, an infinite lifespan, and a stable karyotype composed of 45 chromosomes, including two marker chromosomes. They grow at a moderate rate in medium without exogenous growth factors, but do not form foci or large-sized colonies in 0.33% agarose, and do not form tumors when injected into athymic mice. The cell strain designated MSU-1.1 H-*ras* A2-10T was derived from a round cell sarcoma formed by injection of MSU-1.1 cells transformed by transfection of a plasmid containing the H-*ras* oncogene from the T24 cells under the control of its endogenous promoter.[3] The cell strain designated MSU-1.1-N-*ras* 1 was derived by Wilson *et al.* (9) by transfection of a plasmid containing an N-*ras* oncogene under the control of an LTR promoter.

Culture Conditions. The cells were cultured in McM medium (14), a derivative of MCDB110 basic medium (15) but modified from that described by Ryan *et al.* (14) by being prepared with Earle's salts. Complete medium refers to modified McM containing 10% SCS[4] (Sterile Systems, Logan, UT), penicillin (100 U/ml), and streptomycin

(100 µg/ml) and hydrocortisone (1 µg/ml) to foster attachment to the plastic substrate. The cells were maintained at 37°C in a humidified incubator with 5% CO_2 and the medium was renewed every three or four days.

Plasmids. A fusion gene construct, designated pMT-*ras*T24 (10), was obtained as a gift of Dr. M.W. Lieberman, Baylor University, Houston, TX. The plasmid contains the coding region of the human H-*ras* oncogene fused to the 5' region of the mouse metallothionein I (MT). This fusion construct allows expression of the H-*ras* oncogene to be regulated by varying the concentration of zinc sulfate in the medium. A plasmid containing the metallothionein promoter but lacking the *ras* gene (pMT) was used as a control.

DNA Transfection. Cells were plated into 100 mm diameter dishes at a density of 1 X 10^5 cells/dish in complete medium and incubated at 37°C. After 24 h, the cells were transfected with 2.0 µg of plasmid DNA per dish using the Polybrene/DMSO method as modified by Morgan *et al.* (16).

Assay for Focus-Formation. Following transfection, the cells were allowed a 48 h expression period. Then, the complete medium was exchanged for McM medium containing 1% SCS and antibodies and supplemented with zinc sulfate (50 µM) or not supplemented. The cells were allowed to grow to confluence. The medium was renewed weekly and the dishes were scanned for foci after 3 to 4 wk.

Assay for Anchorage Independence. Cells (5000 per 60-mm-diameter dish) were assayed for their ability to form colonies in 0.33% agarose in modified McM medium containing 2% SCS, as described previously (17).

Growth Factor Independence. The cells' requirement for exogenous growth factors for cell replication were assayed as described (8, 9). In brief, cells (5×10^4) were plated into a series of 60 mm diameter dishes with modified McM medium supplemented with 1% FCS. After 24 h, the number of attached cells was determined in two representative plates by trypsinizing the cells and counting them with a Coulter counter. The medium in the rest of the dishes was changed to McM medium containing 10 % SCS (positive control), or McM medium with 0.1mM calcium and SR_3, or McM medium with 0.1 mM calcium and 0.1% FCS. The cells were re-fed with the appropriate medium every 3 days, and the number of cells in duplicate dishes for each condition was determined 4 and 7 days after plating.

Assay for Tumorigenicity. Long term continuous administration of zinc at a high concentration (greater than $\geq$50 mM) is toxic to athymic mice and so in the initial tumorigenicity study two non-toxic doses were evaluated, 25 and 35 mM concentration. Mature BALB/C athymic males were housed with heterozygous BALB/C females and zinc sulfate was added to the drinking water. When athymic offspring were obtained, they continued to receive the same concentrations of zinc sulphate in their drinking water. Exponentially growing cells were harvested and resuspended in PBS and 1×10^7 were injected subcutaneously into 4 wk old athymic mice. These mice continued to receive zinc in their drinking water for the length of the assay. The mice were observed weekly for

tumor growth, and measurements were made using a venier caliper. Tumors were removed for histologic examination when they were 0.5-1.0 cm in diameter. In order to develop tumor-derived cell lines for further study, portions of the tumor tissues were returned to culture, and the cells were grown in medium containing the antibiotic G418 to eliminate cells of mouse origin. MSU-1.1 cells are resistant to this drug. The volume of tumors was calculated by using the formula for the volume of a sphere. We estimated the radius from the average of two diameters measured perpendicular to each other. Sections of the tumor were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin embedded tumor tissues were then sectioned at 6-8 μm, stained with hematoxylin and eosin, and examined microscopically.

Serum Analysis for Zinc

At necropsy, blood was collected in silicone coated tubes and then placed at room temperature for 12 hours or 4°C overnight. The sera was then removed and placed in separate tubes and stored at -20°C until they were analysed. The serum was analysed at the Animal Health Diagnostic Laboratory, Department of Toxicology, Michigan State University, East Lansing, MI. Determination of serum zinc concentration was courtesy of Dr. Robert Poppenga.

Immunoprecipitation. The amount of p21 *ras* protein synthesized by the cell strains was assayed using immunoprecipitation essentially as described by Wilson *et al.* (9) except that the cells to be assayed were maintained in exponential growth in complete medium containing 0, 25, 50, or 75 μM of zinc sulfate for 3-4 days before labeling or for the

indicated time. The medium was exchanged for 1.5 ml of modified McM medium lacking cysteine and methionine with 1% FCS, penicillin (100 U/ml), streptomycin, 25, 50, or 75 μM of zinc sulfate, and 750 μCi of L-methionine ^{35}S-L-cysteine ^{35}S (Tran ^{35}S-Label; ICN, Irvine, CA) to label the cells for 18 h. All other steps were as described (9). The antibodies used were v-H-*ras* Y13-259 (Oncogene Science, Cambridge, Mass), which reacts with H-, K-, and N-*ras* (10) and monoclonal antibody NEI-701, anti-*ras* p21 (val 12), which reacts specifically with *ras* p21 that has a valine in the 12th codon. The latter was obtained from E. I. Dupont de Nemours, Inc., Boston, MA.

In *vitro* Quantitation of *ras* p21 on the Immunoprecipitation Gels. The intensities of the *ras* p21 bands were quantified using the Bio-Image Visage 110 system (Millipore). The band with a molecular weight of 21kD was outlined, and measurements were taken and the values were corrected by subtracting the background, i.e., the value obtained in a similar area on the film where no band was seen.

Immunohistochemical Detection of *ras* Protein Expression in Tumors. Paraffin-embedded sections of tumors that were 6-8 μm thick were mounted on poly-l-lysine coated glass slides, deparaffinized in xylene, hydrated through decreasing concentrations of ethyl alcohol, and washed in PBS (pH 7.2). After each of the following steps for staining, the slides were washed in PBS. Endogenous peroxidase activity was inhibited by immersing slides for 10 min in absolute methanol containing 30% H_2O_2. Following a 20 min rinse in double-strength PBS, the sections were incubated for 20 min with diluted normal goat serum, the species in which the secondary antibody was made. Excess serum

was blotted from the sections. For 15 min the sections were incubated with monoclonal antibody NEI-701 (Biotechnics, Gettysburg, MD), anti-*ras* p21 (val 12), at a dilution of 1:250. After a 10 min rinse in PBS, the sections were incubated with a biotinylated secondary antibody for 30 min and then with avidin-biotin-peroxidase for 45 min (Vectastain ABC kit, Vector Lab., Burlingame, CA). The sections were incubated with the chromogen 3,3, diaminobenzidine (DAB) for 2 min. The peroxidase DAB reaction forms a stable brown product if the *ras* p21 (val 12) oncoprotein is present. Sections were then lightly counterstained with Gill's hematoxylin, dehydrated, cleared, and mounted with non-aqueous coverslips resin (Permount, Fischer Scientific, Cincinnati, OH).

Quantitation of *ras* Expression in Tumors. Quantitation of steady state levels of the *ras* oncoprotein in tumors was determined by morphometry using a modification of the methods described by Richmond *et al.*, (18), using a Nikon Microphot-FX microscope (Nikon Instrument Group, Oak Park, Illinois) with apochromatic lenses and equipped with a MTI series 68 video camera. The video data were analyzed using a Zenith computer and the JAVA image analysis software version 1.40. Measurements were made randomly using a predefined rectangular area without reference to the contents of each field. A software macro program was used to facilitate the recording and analysis of the data. The analysis involved the computation of the average integrated optical density for each slide.

Statistics. Results are expressed as mean $\pm$ standard deviation. Differences between means were examined using Newman-Keuls multiple comparison test at $P<0.01$.

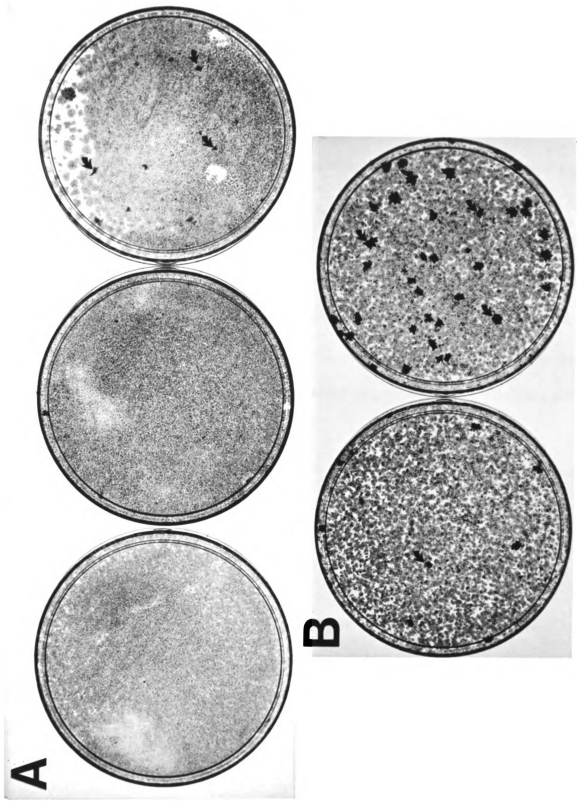
RESULTS

Zinc Dependence of Focus Formation by pMT-*ras*T24 Transfected Cells. Thirty (30) dishes containing $1x10^5$ MSU-1.1 cells were divided into three groups. One group was mock transfected, the second was transfected with the control plasmid, pMT, and the third was transfected with the pMT-*ras*T24 plasmid. Forty-eight hours after transfection, 50 μM of zinc sulfate was added to half the dishes of each group. The medium was renewed weekly. After 3 wk, several dishes from each group were stained with crystal violet and the number of foci were counted. Non-transfected cells and cells transfected with the pMT plasmid grew as a flat monolayer. No foci were observed in the dishes containing these cells with or without the addition of zinc. The MSU-1.1 cells that were transfected with pMT-*ras*T24 but did not receive zinc formed small foci (Figure 2.1A) at a very low frequency (average 1 foci/dish). The population that received zinc formed large foci (Figure 2.1B) and at a higher frequency (average 9 foci/dish).

Eighteen representative foci were isolated from dishes containing pMT-*ras*T24-transfected cells treated or not treated with zinc. These cell populations were expanded and then cloned to assure that each population was pure. Five independent clonal populations that were found to exhibit a morphological change in response to the presence of zinc in the culture medium were selected for further characterization. These were designated MSU-1.1-MT-*ras* 1, MSU-1.1-MT-*ras* 2, etc.

Figure 2.1A: Focus forming ability of MSU-1.1 and MT-H-*ras* transfected MSU-1.1 cells with and without zinc following transfection with MT-*ras*T24. The cells were allowed to grow for 3 wk with (50 μM) or without zinc. (*Left*) MSU-1.1 cells with zinc, (*middle*) MSU-1.1-MT-H-*ras* transfected cells without zinc. (*Right*) MSU-1.1-MT-H-*ras* transfected cells with zinc. Note foci at point of arrow.

Figure 2.1B: Focus forming ability of MSU-1.1-MT-*ras*T24 cells not previously exposed to zinc. Confluent cells not previously exposed to zinc were plated at 33,000 cells per dish with (50 μM) *right*, or without zinc, *left*. The cells were refed 3x weekly with the appropriate media and the dishes stained for foci after 3 wks.


A
B

Effect of Zinc on the Level of Expression of the H-*ras* Oncogene in the pMT-*ras*T24-Transformed Cell Strains. Using the immunoprecipitation technique, the five cell strains were compared for their basal level of *ras* p21 expression in the absence of zinc sulfate in the medium or then in the presence of various concentrations of zinc sulfate. All five pMT-*ras*T24 transformed cell strains had basal levels of expression of the *ras* protein that were higher than that of the parental cell MSU-1.1 cells (Table 2.1). The basal levels in MSU-1.1-MT-*ras* 4 and MSU-1.1-MT-*ras* 5 were almost as high as the level in the MSU-1.1-H-*ras* A2-10T cell strain a cell strain known to express the *ras* oncoprotein at high levels. When zinc was added to the medium, the level of *ras* expression in the five MT-*ras* cell strains increased significantly. As expected, addition of zinc did not increase the level of expression of *ras* p21 in the parental MSU-1.1 cells or in the MSU-1.1-H-*ras* A2-10T cell strain.

Figure 2.2 compares the level of expression of *ras* p21 to that found in the parental MSU-1.1 cells and in two human fibrosarcoma-derived cell lines and an MSU-1.1 cell transformed by a transfected N-*ras* oncogene, and shows the response of cell strains MSU-1.1-MT-*ras* 2 and MSU-1.1-MT-*ras* 3 after 0, 18 or 36 h of incubation in medium containing 50 μM zinc sulfate. The amount of *ras* protein synthesized by MSU-1.1-MT-*ras* 2 and MSU-1.1-MT-*ras* 3 was four and six-fold higher, respectively, than the basal level seen when no zinc sulfate was added to the medium. To determine whether the steady-state *ras* oncoprotein level was proportional to the zinc concentration, three of the

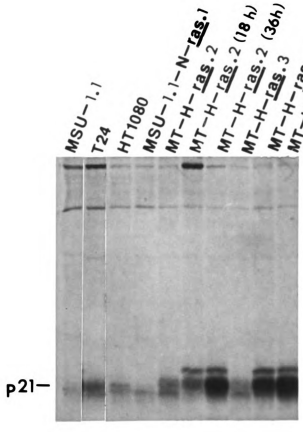
Table 2.1 Relative expression of the *ras* p21 in pMT-H-*ras*T24-transformed MSU-1.1 cells as a function of zinc sulfate concentration

Cell strain	Concentration of zinc sulfate			
	0	25	50	75
MSU1.1	1.0	1.0	1.0	1.0
MSU-1.1-MT-H-*ras*. 1	1.2	2.1	34.9	42.4
MSU-1.1-MT-H-*ras*. 2	2.2	15.3	22.9	18.3
MSU-1.1-MT-H-*ras*. 3	2.6	12.2	27.5	13.5
MSU-1.1-MT-H-*ras*. 4	8.4	[a]ND	ND	28.5
MSU-1.1-MT-H-*ras*. 5	10.0	ND	ND	88.0
[b]MSU-1.1-H-*ras*. 10T	12.0	12.1	12.0	12.0

[a]Not determined.

[b]This cell strain was transformed by transfection of an *H-ras* oncogene driven from its endogenous promoter, and therefore should not respond to the presence of zinc in the medium. Cells were grown in modified McM medium with 1% FCS for 3-4 days with 0, 25, 50 and 75 μM of zinc sulfate, labeled with L-methionine ^{35}S-L-cysteine ^{35}S for 18 hrs and analyzed for total *ras* expression by immunoprecipation with antibody Y13-259, which reacts with all forms of *ras* p21 as described above. Quantitation of the amount of protein on gels is described in the Materials and Method Section. The level of *ras* p21 in the parental MSU-1.1 cells in the presence or absence of zinc sulfate was arbitrarily designated 1.0.

Figure 2.2: Analysis of *ras* gene expression 0, 18 or 36 h after exposure to 50 μM of zinc. Trans ^{35}S labeled cellular extracts were immunoprecipitated with the monoclonal antibody Y-13-259, the immunoprecipates were electrophoresed on 12.5% polyacrylamide gels, and the gels were analyzed by fluorography. *Lane 1* negative control, parental cell line (MSU-1.1). *Lanes 2-4* (positive controls), *Lanes 4-10* cells transfected with MT- *ras* T24 (p21 molecular weight of *ras* proteins).


MSU-1.1
T24
HT1080
MSU-1.1-N-ras.1
MT-H-ras.2
MT-H-ras.2 (18 h)
MT-H-ras.2 (36h)
MT-H-ras.3
MT-H-ras.3 (18h)
MT-H-ras.3 (36h)
p21-

MT-*ras*T24-transformed cell strains were grown in medium containing various concentrations of zinc for 4 days and assayed for *ras* as above. The level of *ras* p21 expression is proportional to the concentration of zinc in the medium (Figure 2.3A). It was not possible to assay the level of expression in cells exposed to 100 μM of zinc, since most of the cells detached from the dishes. This was probably related to the high levels of *ras* p21 expression since MSU-1.1 H-*ras* A2-10T cells did not detach in medium containing this concentration of zinc. Toxicity caused by high expression of the *ras* oncoprotein has been previously observed in MSU-1.1 cells in this laboratory[3], as well as in rat fibroblasts (19).

Figure 2.3B shows the response of the MSU-1.1-MT-*ras* 4, and MSU-1.1-MT-*ras* 5 cell strains when 75 μM zinc sulfate was added to the culture medium. Both showed a marked increase in the amount of *ras* oncoprotein. As expected, MSU-1.1 and MSU-1.1-H-*ras* A2-10T cells did not show any increase in expression in response to 75 μM zinc.

Influence of the Concentration of Zinc in the Medium on Cell Morphology. To examine the effects of increased expression of the H-*ras* oncogene on cell morphology, we plated 5 x 10^5 cells into a series of 60 mm diameter dishes in complete medium. After 24 h, 0, 25, 50, 75, or 100 μM zinc sulphate was added. All the plates were observed daily, and were photographed with an inverted microscope on day 4 or 5. MSU-1.1-H-*ras* A2-10T cells, which constitutively express the *ras* gene at high levels, was used as a positive control, and parental MSU-1.1 cells were used as a negative control. When grown in medium without added zinc, the five pMT-*ras*T24-transformed cell strains exhibited

Figure 2.3A: Analysis of *ras* gene expression after 4 d of continuous exposure to zinc at different concentrations. Trans ^{35}S labeled cellular extracts were immunoprecipated with the monoclonal antibody Y-13-259, the immunoprecipates were electrophoresed on a 12.5% polyacrylamide gel. Gels were analyzed by fluorography.

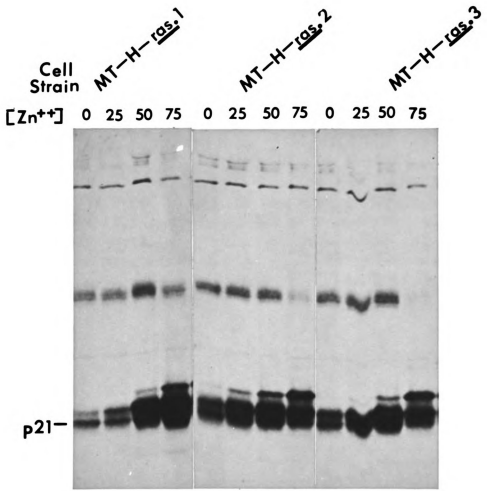

Cell Strain
MT-H-ras.1
MT-H-ras.2
MT-H-ras.3
[Zn++] 0 25 50 75 0 25 50 75 0 25 50 75
p21-

Figure 2.3B: Effect of zinc on *ras* gene expression in the parental cell strain MSU-1.1 (negative control) H-*ras* 10T (positive control) and cell strains MT-*ras* 4 and 5. Trans ^{35}S labeled cellular extracts were immunoprecipated with the monoclonal antibody Y-13-259. The immunoprecipates were electrophoresed on a 12.5% poly acrylamide gel. Gels were analyzed by fluorography. *Lanes 1 and 2* negative control, *Lanes 3 and 4* positive control, *Lanes 5-6*, MT-*ras* 4, *Lanes 7-8*, MT-*ras* 5.

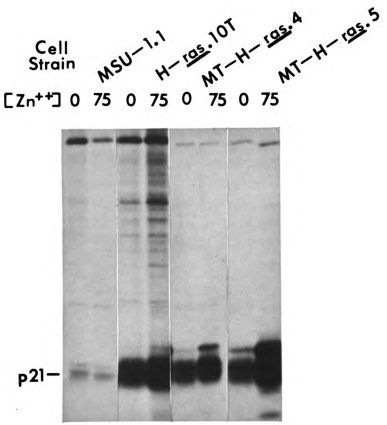
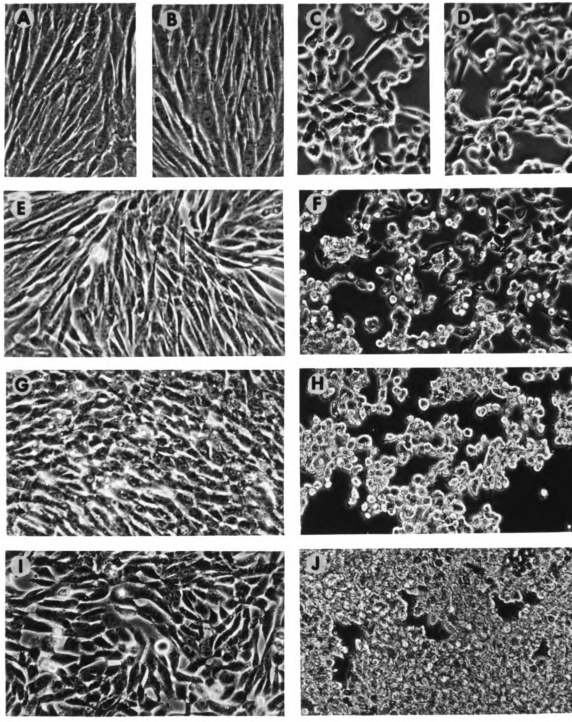

Cell Strain
MSU-1.1
H-ras.10T
MT-H-ras.4
MT-H-ras.5
[Zn++] 0 75 0 75 0 75 0 75
p21-

Figure 2.4: Morphology of MT-*ras* transfected cells in response to different concentrations of zinc in the culture medium. Parental cell line (A-B) A, 0 zinc; B, 75 μM of zinc. A2-10T, H-*ras* transformed cell strain (C-D) C, 0 zinc; D, 75 μM of zinc. MT-H-*ras* 1; (E-F) E, 0 zinc; F, 75 μM zinc. MT-H-*ras* 3, (G-H) G, 0 zinc; H, 75 μM of zinc and MT-H-*ras* 5, (I-J) I, 0 zinc; J, 75 μM of zinc.


A
B
C
D
E
F
G
H
I
J

various degrees of morphological transformation that correlated with their basal level of *ras* expression. The parental cell strain, MSU-1.1, and MSU-1.1 H-*ras* A2-10T cell strain exhibited no change when exposed to 75 μM of zinc (Figure 2.4; A-D). Cell strains MSU-1.1-MT-*ras* 1 and MSU-1.1-MT-*ras* 3, which expressed low basal levels of *ras* and MSU-1.1-MT-*ras* 4 and MSU-1.1-MT-*ras* 5, which expressed moderately high basal levels, (Table 2.1) exhibited corresponding degrees of morphological transformation (Figure 2.4; E, G and I). When 25 μM of zinc was added to the medium, no change in morphology was noted. However, concentrations of zinc of 50 μM or more caused profound morphological changes. For example, with addition of 75 μM of zinc, the cells became more refractile and rounded or epitheloid (Figure 2.4; F, H and J).

Zinc Induced Changes in Cellular Morphology are Reversible. To determine whether the morphological changes induced by zinc were reversible, MT-*ras* transformed cells were exposed to 25, 50, and 75 μM of zinc for four days, and then they were either trypsinized and replated in medium lacking zinc or were re-fed with complete medium without zinc. After three to five days, the cells had morphologic features and a growth pattern indistinguishable from MSU-1.1-MT-*ras* cells that had not been exposed to zinc. Seyama *et al.* (20) and Reynolds *et al.* (10) found similar results with rodent liver epithelial cells and rat fibroblasts respectively when they carried out such studies.

Anchorage Independence of MT-*ras* Transfected Cells. To determine the effect of basal expression levels of the H-*ras* oncoprotein on anchorage-independent growth, the MT-*ras* T24-transformed cell strains were assayed for ability to form large-sized colonies

Table 2.2 Relationship between the level of *ras* expression in pMT-*ras*T24 transformed MSU-1.1 cells and their ability to form large-sized colonies in 0.33% agarose

Cell strain	Relative level of *ras* p21[a]	Mean no. of colonies per dish
MSU-1.1	1.0	0
MSU-1.1-MT-H-*ras*. 1	1.2	10
MSU-1.1-MT-H-*ras*. 2	2.1	20
MSU-1.1-MT-H-*ras*. 3	2.6	45
MSU-1.1-MT-H-*ras*. 4	8.4	115
MSU-1.1-MT-H-*ras*. 5	10.0	155
MSU-1.1-H-*ras* .10T	12.0	225

[a]Data taken from Table 2.1

After 3 weeks of growth, the number of colonies with a diameter equal to or greater than 200 μm in duplicate dishes was determined using an automated image analyzer. Cells (5000 per dish) were assayed for their ability to form colonies in modified McM medium supplemented with 2% SCS, antibiotics and 0.33% agarose.

in 0.33% agarose. The results (Table 2.2) indicated that the ability to grow in agarose correlates with the cells' basal level of *ras* expression. When zinc was added to the culture medium containing agarose, to induce *ras* synthesis, zinc sulfate interfered with the "gelling" of the agarose so the effect of increased *ras* synthesis on cell growth in agarose could not be determined using this system.

Growth Factor Requirements of MSU-1.1-MT-*ras* Cells. Diploid human fibroblasts cannot replicate in McM medium containing 0.1 mM Ca^{2+} supplemented with SR_3, without the addition of protein growth factors such as EGF, PDGF or bFGF. In contrast, *ras*-transformed, MSU-1.1 cells or human fibrosarcoma-derived cell lines do not require such growth factors (8, 9). To determine if pMT-*ras*T24-transformed cell strains could replicate without such exogenous growth factors, and whether this phenotype was dependent on the level of expression of the *ras* oncoprotein, we compared cell strains (MT-*ras*-1, MT-*ras* 2 and MT-*ras* 3) that expressed different basal levels of the H-*ras* oncoprotein for their ability to replicate in McM medium containing 0.1 mM calcium and supplemented with SR_3 and 0.1% FCS. The data indicated that all three experimental cell strains and the A2-10T cells replicated rapidly under these conditions, whereas the parental cell strain MSU-1.1 replicated only minimally (Figure 2.5). There was no significant difference in the rate of replication of the three experimental transformed cell strains tested.

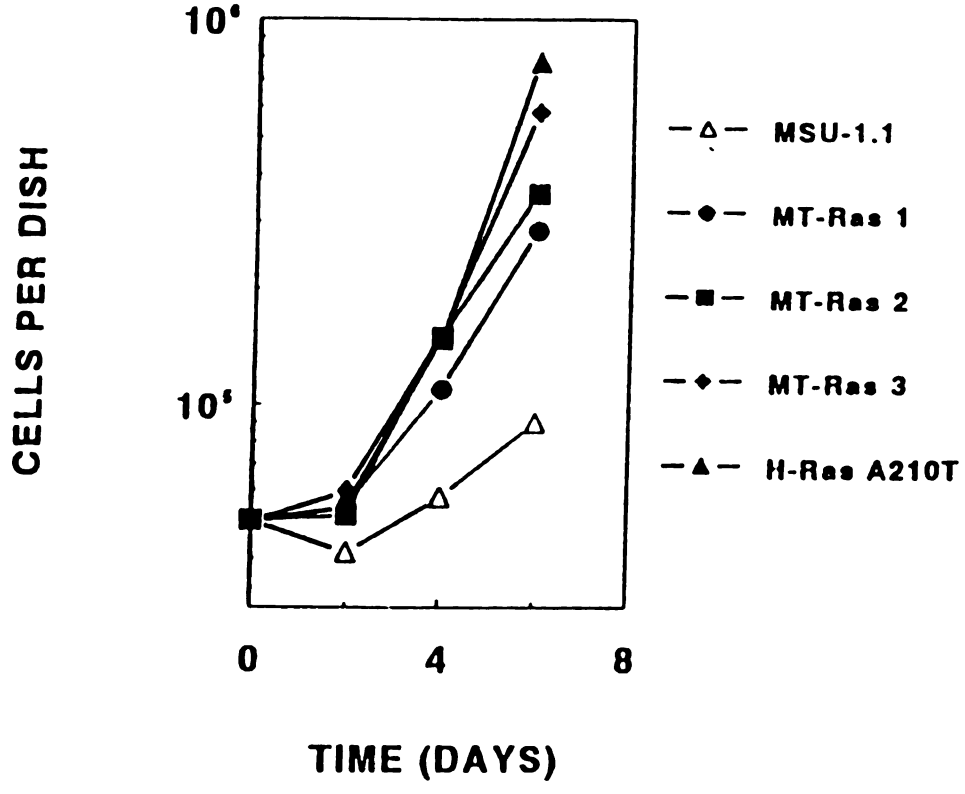


Figure 2.5: Growth factor requirements of MSU-1.1 cells, and cell strains H-*ras* 10 A2T and MT-*ras* 1, 2 and 3. Cells were plated in McM medium with 1% FCS. The next day, the number of cells attached was determined, and the medium was replaced with McM medium modified to contain only 0.1 mM calcium and supplemented with SR_3 and 0.1% FCS. Cells were refed on day 4 and counted on days indicated.

Tumorigenicity of Primary MT-*ras* Transformed Cells. Zinc supplementation produced serum zinc levels of 28-32 μM in the offspring of athymic mice, which were exposed continually to zinc sulfate in their drinking water during pregnancy, while zinc levels were 3-5 μM in the offspring of mice that were not given zinc. Exponentially growing, pMT-*ras*T24-transformed cells were assayed for tumorigenicity by subcutaneous injection into athymic mice that were exposed to zinc sulfate in their drinking water or to mice that were not exposed to zinc. H-*ras* A2-10T cells were injected as a positive control.

MT-*ras* 2 transformed cells were non-tumorigenic and MT-*ras* 5 transformed cells formed tumors with a latency period of 3-4 weeks regardless of the mice zinc status. If the two long latency MT-*ras* 1 tumors in control mice are disregarded, MT-*ras* 1, 3 and 4 transformed cells formed tumors only in mice given zinc (Table 2.3). These tumors developed after a latency period of 3-4 weeks. Low (MT-*ras* 1) or intermediate (MT-*ras* 3) expression of the *ras* oncoprotein was associated with tumorigenicity in mice given high zinc, 50 mM (Table 2.3). In contrast, high *ras* expression (MT-*ras* 4) was associated with tumorigenicity at low and high zinc levels and at very high levels of *ras* expression (MT-*ras* 5) tumors formed with a high frequency even in mice not given zinc. For MT-*ras* 5 there was no difference in the average latency period for the tumors formed in mice supplemented with or without zinc. Regardless of the zinc status of the mice, MSU-1.1-MT-*ras* 5 transformed cells which expressed the *ras* protein at very high levels, formed

Table 2.3 Effect of zinc sulfate on the incidence, latency period and types of tumors formed by primary MSU-1.1-MT-H-*ras* transformed cells

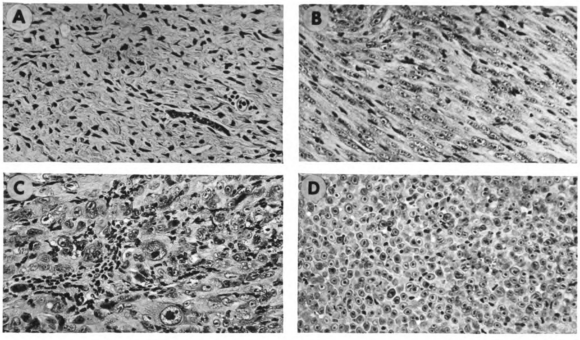
Cell Strain	Zinc concentration (mM)	Tumor incidence	Latency[a] days	Tumor histology
MSU-1.1-MT-H-*ras* 1	0	2/16	195	Mixed sarcoma
	25	0/22	--	--
	35	7/10	21	Fibroma
MSU-1.1-MT-H-*ras* 2	0	0/8	--	--
	25	0/16	--	--
	35	0/4	--	--
MSU-1.1-MT-H-*ras* 3	0	0/12	--	--
	25	0/6	--	--
	35	3/4	32	Spindle cell sarcoma
MSU-1.1-MT-H-*ras* 4	0	0/8	--	--
	25	4/4	23	"Pleomorphic sarcoma"
	35	6/10	25	"Pleomorphic sarcoma"
MSU-1.1-MT-H-*ras* 5	0	7/8	21	Round cell sarcoma
	25	3/4	19	Round cell sarcoma
	35	4/6	26	Round cell sarcoma
MSU-1.1-H-*ras* 10T	0	4/4	7	Round cell sarcoma
	25	ND	--	--
	35	ND	--	--

[a]Time until tumor reached a diameter ≥3 mm. ND, Not done; Athymic males were housed with heterozygous females with 35mM of zinc sulfate in drinking water. The athymic offsprings from these matings were injected subcutaneously with $1x10^7$ exponentially growing cells. The mice were observed for tumors weekly and measurements taken with a venier caliper. All tumors were removed and examined microscopically.

multi-nodular, subcutaneous masses that on gross examination were well vascularized. Histologically, these were high grade malignant tumors with no difference in the histology of tumors from mice that received zinc versus those that did not (Table 2.3). The tumors consisted of a pleomorphic population of round to polygonal cells with large, multiple, prominent nucleoli, and a high mitotic index. The tumors also contained numerous blood vessels (Figure 2.6D). In contrast, in mice given zinc, the low expressing MT-*ras* 1 transformed cells consistently formed benign tumors. These tumors were primarily small, hard, subcutaneous growths composed of well-differentiated fibroblasts in a loosely arranged pattern (Figure 2.6A). No mitotic figures were observed. Two mixed sarcoma formed by MT-*ras* 1 transformed cells in control mice were clearly different from their tumor counterparts formed in mice given zinc. Because of the long latency and the marked difference in histology of the tumors that developed in mice that were not given zinc, we attribute the growth of these tumors to an additional spontaneous change that arose in the cell population.

MSU-1.1-MT-*ras* 3 cells, which have intermediate levels of induced *ras* expression, consistently formed spindle cell sarcomas (Figure 2.6B). These tumors consisted of broad interlacing fascicles of poorly differentiated fibroblasts with four to six mitotic figures per 40X field. Cell strain MSU-1.1-MT-*ras* 4 induced malignant tumors (pleomorphic sarcomas) that consisted of large, "strap-like" cells with one or two nuclei and abundant cytoplasm i.e., had histologic features of rhabdomyosarcomas (Figure 2.6.C).

Figure 2.6: Photomicrograph of formalin fixed, hematoxylin/eosin stained tissue sections of tumors formed from injection of MT-H-*ras* transformed cell strains. (A) Benign tumor (fibroma) produced by cell strain MT-H-*ras* 1 (x100). Note well differentiated stellate fibroblasts, and low mitotic index. (B) High grade malignant spindle cell sarcoma "Fibrosarcoma" (x100) produced by cell strain MT-H-*ras* 3; (C)"Pleomorphic sarcoma" (x100) with large "strap-like cells" characteristic of Rhabdomyosarcoma produced by MT-H-*ras* 4. (D) High grade malignant "Round cell sarcoma" (x100) produced by cell strain MT-H-*ras* 5.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Expression of the *ras* Oncoprotein in MSU-1.1-MT-*ras* Primary Tumors Derived from Mice Supplemented with Zinc. Since the phenotypes of four MSU-1.1-MT-*ras* tumors were different we wished to determine whether there there was a correlation between *in vivo* expression of the *ras* oncogene and the tumor phenotype. To address this question we conducted an immunohistochemical study. Tumors formed by H-*ras* A2-10T cells, an MSU-1.1 cell strain that constitutively express the $p21^{val}$ oncoprotein was used as a positive control and tumors formed by the MSU-1.1-v-*sis* cells, a strain that does not express the *ras* oncoprotein was used as a negative control for immunostaining. Table 2.4 summarizes the results of the relative expression of the *ras* oncoprotein in these MSU-1.1-MT-*ras* induced tumors as determined by immunohistochemical staining and morphometry (image analysis). There is a qualitative agreement between the *in vivo* expression levels with the data *in vitro* as shown in Table 2.1.

Figures 2.7 and 2.8 show the results of immunohistochemical staining of tumors for the H-*ras* oncoprotein using the $p21^{val}$ antibody, which is specific for the transfected oncogene protein. A tumor formed by v-*sis* transformed MSU-1.1 cells served as a negative control and it shows no staining for the H-*ras* oncoprotein (Figure 2.7A). Figure 2.7B shows the immunohistochemical staining for the H-*ras* oncoprotein in tumors induced by MT-*ras* 1 cells. There is positive cytoplasmic staining for the *ras* protein. Figure 2.8C shows the immunohistochemical staining of a fibrosarcoma formed by MSU-1.1-MT-*ras* 3 transformed cells. In this tumor the cells exhibit strong positive cytoplasmic and

Table 2.4 Relative expression of the *ras* p21 protein in primary tumors induced by MT-H-*ras* transformed cells and formed in zinc-treated mice.

Cell strain	Relative *ras* expression	
	Range	Mean ± SD
*v-*sis* Tumor	0.082 - 0.087	0.085 ± 0.001
**H-*ras*. 10T	0.431 - 0.461	0.441 ± 0.004
MT-*ras*. 1	0.182 - 0.189	0.186 ± 0.002
MT-*ras*. 3	0.279 - 0.296	0.285 ± 0.005
MT-*ras*. 4	0.325 - 0.358	0.344 ± 0.013
MT-*ras*. 5	0.501 - 0.556	0.526 ± 0.019

*Negative control **Positive control

Values represent the average integrated optical density (IOD) for ten (10) fields in 2-5 tumors per cell strain. Results are expressed as the means and standard deviation (SD); all MT-*ras* mean values were significantly different from each other and from the controls at $P < 0.01$ using Newman and Keuls multiple comparison test.

Figure 2.7: Immunohistochemistry for *ras* $p21^{val}$ protein in tumors induced by transformed MT-H-*ras*-MSU-1.1 cells. (A-B) v-*sis* tumor (x200) negative control, MT-H-*ras* 1, (C-D) C, low magnification (x100) and D, high magnification (x200). Note the brown staining (positive reactivity) in the cytoplasm of these well differentiated fibroblasts. MT-H-*ras* 3, (E-F) E, low magnification (x100) and F, high magnification (x200).

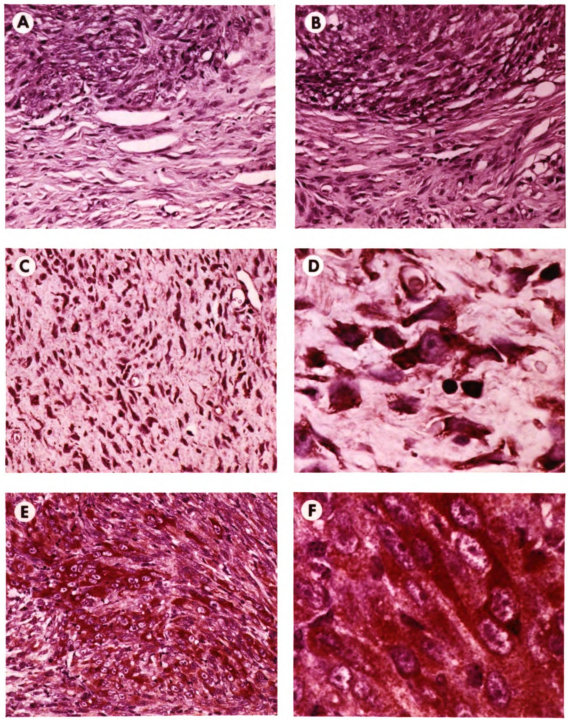
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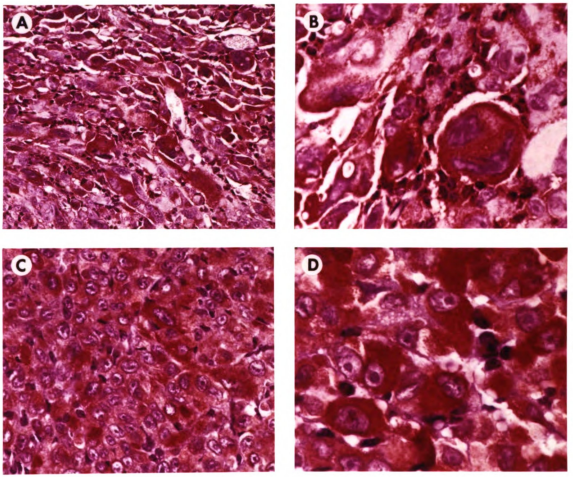
Figure 2.8: Immunohistochemistry for *ras* $p21^{val}$ protein in tumors induced by transformed MT-H-*ras*-MSU-1.1 cells. MT-H-*ras* 4, (C-D) C, low magnification (x100) and D, high magnification (x200). Note the brown staining (positive reactivity) in the cytoplasm of these well differentiated fibroblasts.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membrane immunohistochemical staining for the *ras* oncoprotein. The malignant tumors formed by MSU-1.1-MT-*ras* 2, 3, 4, and 5 transformed cells exhibited more intense positive staining for the *ras* oncoprotein than did the benign tumor formed by MSU-1.1-MT-*ras* 1 transformed cells.

Growth of Primary MT-*ras* Tumors in Mice Supplemented with Zinc. We compared the growth rate of the MSU-1.1-MT-*ras* tumors to determine if the level of expression of the H-*ras* oncogene correlated with the rate of tumor growth. The growth rate of the tumors induced by MSU-1.1-MT-*ras* 3, and 4 cell strains were very similar and so MSU-1.1-MT-*ras* 4 is considered typical of this group. There were marked differences in the rate of tumor growth for cell strains MSU-1.1-MT-*ras* 1, 4, and 5 (Figure 2.9). In general all tumors grew progressively. The MT-*ras* 5 cell strain (which constitutively expresses the *ras* oncoprotein at high levels) induced tumors that appeared well vascularized, grew progressively and were the largest of the group. The data clearly indicate that the high basal level of expression of the H-*ras* oncoprotein (Table 2.4) correlates with rapid tumor growth (Figure 2.9).

Effect of Zinc Withdrawal on Growth of Tumors Induced by MT-*ras* Transformed Tumor-Derived Cells. Since (as shown above) cells that were morphologically transformed in the presence of zinc, reverted to normal morphology when zinc was not present in the medium, we were interested in determining the response of tumors in mice when the drinking water with added zinc was replaced with water with no added zinc.

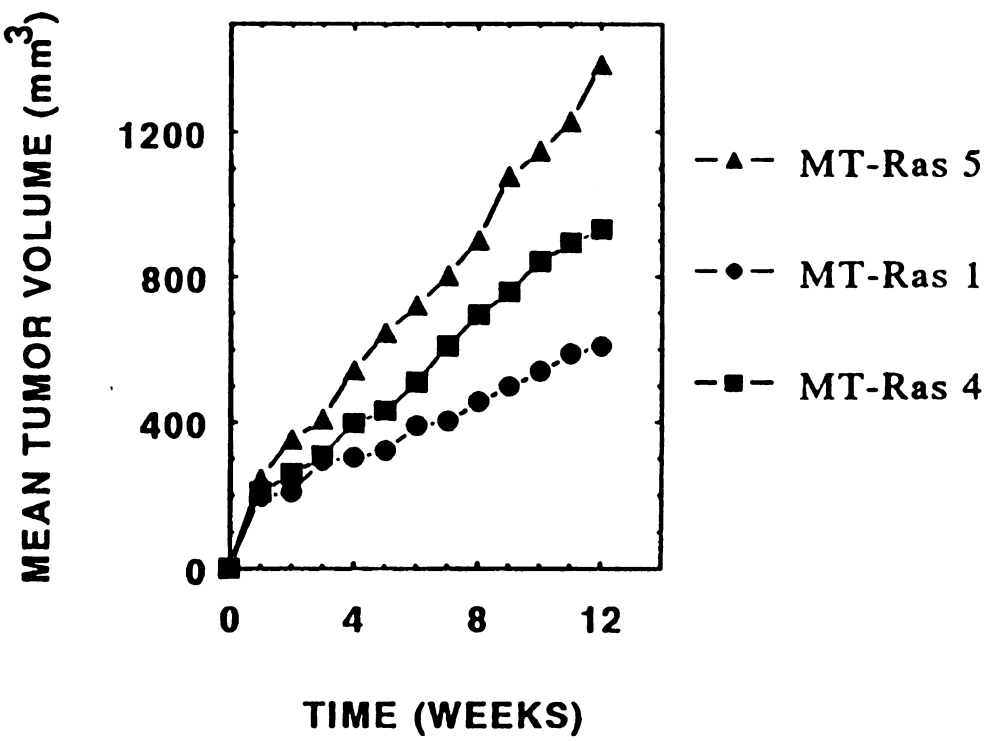

Figure 2.9: Growth rate of tumors induced by cell strains MT-H-*ras* 1, 4 and 5. Tumor volume was calculated using the formula for the volume of a sphere. Measurements were taken weekly and recorded.

This was initially planned as a part of the tumor experiment described but errors in experimental design and carrying out the protocol necessitated repeating this phase of the study. The mouse colony at Michigan State University had to be sacrificed because of a bacterial infection and so the zinc withdrawal phase of the study was sent out to a contract laboratory. This study was conducted with the assistance of Drs Martin Wenk and David Jacobson Kram at Microbiological Associates Rockville MD. Because of changes in the zinc sulfate formulation the protocol used by Microbiological Associates was modified in two ways (1) change in the methodology of zinc administration (2) use of tumor-derived cells. Briefly, following a quarantine period of at least 14 days mice were given drinking water supplemented with zinc sulphate (50 mM) during the first week followed by the lower concentration of 16 mM. This dosing regimen (16 mM zinc in the drinking water) was continued for approximately 2 weeks after which time the mice were administered drinking water supplemented with 30 mM zinc. The mice received drinking water supplemented with 30 mM of zinc for at least one week before being assigned to the study. For the duration of the study, an additional group of mice was administered drinking water not supplemented with zinc. Prior to injection, all mice were randomized and transformed cells from the MT-*ras* cell strain was injected into six (6) mice; four (4) were from the group fed drinking water supplemented with zinc and the other two (2) mice were fed water not supplemented with zinc.

Tumor-derived cells were utilized for this "withdrawal" phase of the study because re-injected tumor-derived cells typically have a higher incidence of tumors. Furthermore, the

MT-*ras* tumor-derived cells utilized were the progeny of cells that formed tumors in athymic mice supplemented with zinc. Exponentially growing, tumor-derived, zinc-treated (50 μM), MT-*ras* transformed cells, were harvested and resuspended in PBS and $1x10^7$ cells were injected subcutaneously (2 sites per mouse) into athymic mice approximately 7-8 weeks of age. When the tumors were at least 3 mm in diameter (approximately 6 weeks after injection), two (2) mice (with at least one palpable/visible tumor) from each group (MT-*ras*-1, and MT-*ras* 5) were randomly selected and the drinking water (previously supplemented with 30 mM) switched to water without added zinc while the other two (2) mice continued to receive drinking water supplemented with zinc (30 mM).

As expected, most MT-*ras* tumor-derived cells induced tumors with a shorter latency when compared to the tumors formed from the inital injection of the primary transformed cells. Surprising and unexpectedly, MT-*ras* 3 tumor-derived cells did not induce tumors and the reason (s) for this observation is not known. In general, zinc-treatment had no effect on the incidence of tumors induced by MT-*ras* tumor-derived cells. For all cell strains evaluated, there were no observable and consistent differences in the pattern of tumor growth in mice that received zinc versus those mice that did not receive zinc (Figure 2.10). The latency and incidence of tumorigenicity with and without zinc after injection of the MT-*ras* tumor-derived cells is shown in Table 2.5.

Withdrawal of zinc had no effect on the growth pattern of tumors in athymic mice injected with MT-*ras* 1 and 5 transformed cells (Figure 2.10). At the six week time point (time of zinc withdrawal) MT-*ras* 4 transformed cells did not induce tumors (in the zinc

Table 2.5 Effect of zinc sulfate on the incidence, latency period and types of tumors formed by MSU-1.1-MT-H-*ras* tumor-derived cells.

Cell Strain	Zinc concentration (mM)	Tumor incidence	Latency[a] days	Tumor histology
MSU-1.1-MT-H-*ras* 1	0	2/2	15	Fibroma
	30	3/4	15	Fibroma
MSU-1.1-MT-H-*ras* 3	0	0/2	--	--
	30	0/4	--	--
MSU-1.1-MT-H-*ras* 4	0	2/2	14	"Pleomorphic sarcoma"
	30	4/4	14	"Pleomorphic sarcoma"
MSU-1.1-MT-H-*ras* 5	0	1/2	35	Round cell sarcoma
	30	3/4	21	Round cell sarcoma

[a]Time until tumor reached a diameter $\geq$ 3 mm. Athymic male mice were either treated or not treated with zinc in their drinking water (see Materials and Methods). All mice were injected subcutaneously with exponentially growing, zinc-treated MT-*ras* tumor-derived cells. The mice were observed for tumors and measurements taken weekly. At the end of the experimental period all mice were sacrificed and the tumors removed for microscopic evaluation.

treated group) of sufficient size that these tumors could be included for evaluation in the regression phase of the study. Histologically, the tumor phenotype for the most part was generally consistent i.e., tumor-derived and primary and MT-*ras* transformed cells formed tumors that were similar when they were evaluated histologically.

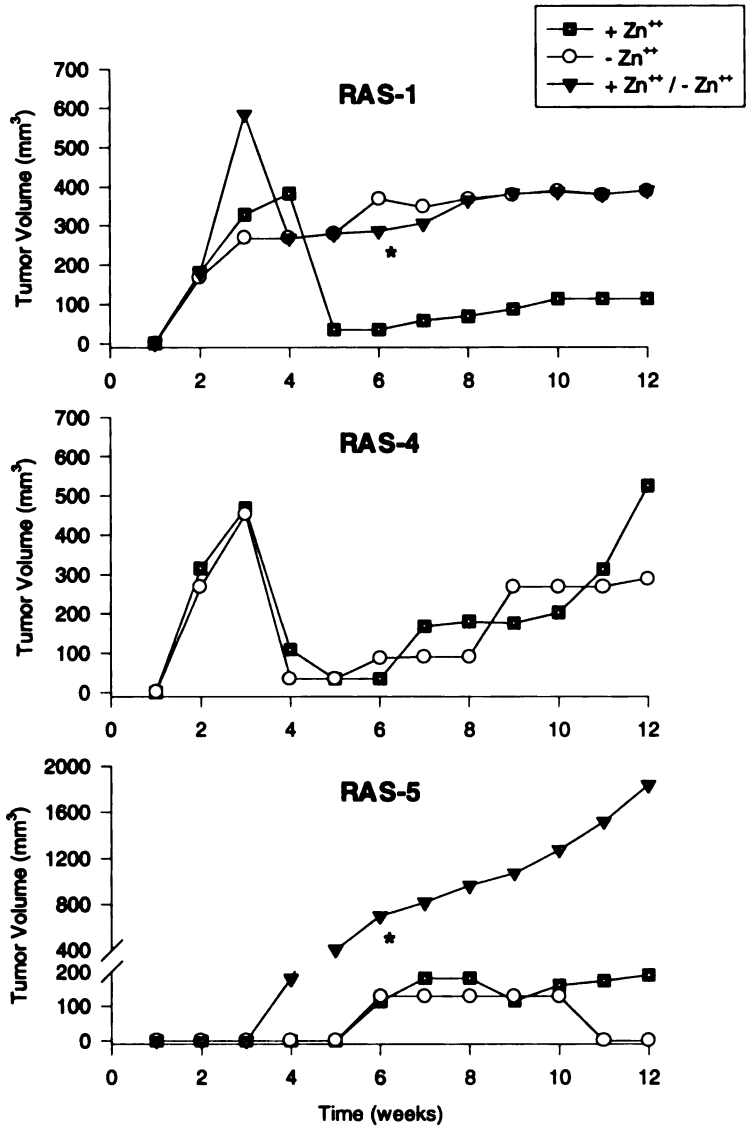

Figure 2.10: Graphical sketch of growth pattern shown as average tumor volume of MT-*ras* tumors with and without zinc. The the effect of zinc supplementation /withdrawal on the pattern of tumor growth was evaluated in mice injected with transformed cells from MT-H-*ras* 1, 4 and 5 cell strains. Zinc was removed (*) from the drinking water of tumor bearing mice 6 wks after injection. Tumor volume was calculated using the formula for the volume of a sphere. Measurements were taken weekly and recorded.

Discussion

We have demonstrated that increasing the steady state levels of the H-*ras* oncoprotein in MSU-1.1 cells correlates with the degree of morphologic transformation, the size of colonies formed in soft agarose, the rate of tumor growth and the histology grade of the tumor indicating that these phenotypes are mediated by the level of the *ras* oncoprotein. Our findings are similar to those reported by Reynolds *et. al.* (10) with Rat-1 fibroblasts. However, they differ from the study by Reynolds *et al.* (10) because they did not report any tumorigenicity data. Another similar study to ours is that of Seyama *et al.* (20) who utilized a *ras* metallothionien containing plasmid with rat liver epithelial cells. These studies are markedly different from ours however, in that all the transformed cells studied induced highly malignant tumors regardless of the level of *ras* oncoprotein expression.

One important result of the present study is that MSU-1.1-MT-*ras* transformed cells induce tumors whose histology grade correlate with expression levels of the *ras* oncoprotein. These data indicate that activating mutations in the H-*ras* gene can play a causal role in the different types of soft tissue tumors observed in humans. However, the expression level of the *ras* oncoprotein or other changes in the pathway in which the *ras* protein is a member, the *ras-raf-MAPK* pathway, may determine the histological grade of the tumor.

Although one could not identify the progenitor cell of a round cell sarcoma as a fibroblast, other studies with the MSU-1.1 cells demonstrate that very high expression levels of the N-*ras* or H-*ras* oncoprotein in MSU-1.1 fibroblasts have reproducibly

produced such tumors when the cells were injected into athymic mice (8, 9). The finding that the MSU-1.1 cell produced rhabdomyosarcomas was surprising. Further studies are needed to determine whether these tumors express specific striated muscle cell markers such as myoD (21). It should be noted however, that mouse fibroblasts can produce tumors which have pleomorphic cells which include "strap cells" (myofibroblasts) so the present observations are not without precedent (22, 23).

Another important result of the present study is that when MSU-1.1-MT-*ras* transfected cells are treated with zinc in culture the transfected cells exhibit features of transformation. However *in vivo*, there was no consistent and reproducible effect of zinc on tumor development or the pattern of tumor growth in the athymic mice treated with zinc and then injected with these transformed cells. The two tumorigenicity studies produce different results as a "clear" zinc effect was observed in the initial experiments but these results could not be repeated using the modified tumorigenicity protocol. In all these studies with zinc, our assumption was that serum zinc concentrations would parallel extracellular interstitial zinc. Therefore, serum zinc levels could be used as a surrogate marker for "free" interstitial zinc and if free zinc were high then this would be sufficient to cause strong activation of the MT promoter in the injected MT-*ras* cells, initiate transcription of the MT-*ras* fusion gene *in vivo* and cause marked elevation of the *ras* oncoprotein which would lead to tumorigenicity. The exact reason why the MT-*ras* transformed cells *in vivo*, exhibited such a inconsistent response to zinc supplementation is unknown, even though in mice supplemented with zinc serum zinc levels were markedly

elevated. We can speculate that there was marked variation and/or marginal increase in interstitial free zinc and this low level of free zinc was inadequate for strong consistent activation of the MT promoter. Evidence to support this as a possible explanation for the failure of the experiment comes from the fact that serum zinc levels are not reflective of tissue or interstitial fluid levels (23) and therefore, our intial assumption was incorrect as we were unaware of these data. Henkin 1971, (24) reported that 80 percent of zinc in plasma is protein bound and only 20 percent circulate free. Therefore, it is likely that in the interstitial fluid compartment only the unbound zinc (20%) is truly "free" and available because proteins (which bind 80%) are not found in appreciable quantities in the interstitial fluid compartment. Thus, the desired consistent elevation in expression levels of the *ras* oncoprotein that was needed to significantly influence tumor growth *in vivo* was lacking. The report by Shaw *et al.* 1992, (25) also support this as a possible mechanism since they achieved strong *in vivo* induction of the MT promoter using local daily injections of 100μM zinc chloride subcutaneously in the skin close to the site where tumor cells were injected. Strong activation of the MT promoter was probably achieved because of the high concentration of free zinc locally in the interstitial fluid in the skin at the site of injection. In our studies, plasma zinc concentrations was ~30μM most of which is expected to be protein bound and therefore unavailable. In the present study, the tumorigenic response exhibited by some MT-*ras* strains may reflect individual variation and it is reasonable to conclude that for the most part the tumors observed in these studies were induced by the basal level of *ras* expression in these MT-*ras* transformed cells.

Previous studies (8, 9) in the Carcinogenesis Laboratory examined the relationship between malignant transformation, tumorigenicity and expression of the *ras* oncoprotein at high levels. Recent studies show that in *ras* transformed malignant tumor cells, inhibition of protein farnesylation (a process which regulates transport of cytoplasmic *ras* protein to the cell membrane) causes malignant tumor cells to revert to a less anaplastic phenotype (26). It has been shown that *ras* transformed cells have the ability to stimulate production of angiogenic factors which result in blood vessel growth *in vivo* (27). It is well recognized that a viable blood supply is an essential requirement for expansion of the tumor mass (28-32). Since angiogenesis is important in maintenance and expansion of the tumor mass, a low level of free zinc in the interstitial fluid zinc *in vivo* may account for the lack of strong activation of the MT promoter resulting in low *ras* oncoprotein expression and hence the inability of potential tumor cells to produce or induce a continuous stimulation and sustainment of angiogenic factors which are required for tumor growth. This hypothesis may provide an explanation for the rapid initial growth of tumors induced by MT-*ras* 1 and MT-*ras* 4 cells which can be attributed to a "carry-over" effect of the zinc. This growth was short lived because the high concentration of free zinc in the culture medium caused a marked increase in *ras* expression which favored tumor growth (by stimulating angiogenic factors) versus the low level of free zinc in the interstitial fluid of athymic mice supplemented with zinc. Hence, the rapid decline in growth of these MT-*ras* 4 tumor cells may be linked directly to down regulation of *ras* oncoprotein expression, and reduction and/or loss of the angiogenic stimuli *in vivo*. Furthermore, the results of

one study (27) suggested a causative relationship between expression of the *ras* oncoprotein and the up-regulation/induction of angiogenic factors such as vascular endothelial growth factor/ vascular permeability factor (VEGF/VPF). More studies are needed however to substantiate these claims. It is interesting to note that in the present study "marked neo-vascularization" was observed histologically in highly malignant tumors induced by transformed cells that express the *ras* oncoprotein at high levels. Additional evidence in support of the "*ras* angiogenesis" hypothesis comes from the work of Thompson *et al.*, (33). They found that RNA tumor viruses carrying the *ras* oncogene caused a greater than 10 fold increase in angiogenesis when compared to similar viruses without the *ras* gene. In addition, the induction of the angiogenic phenotype was concentration-dependent i.e., when 0.01% of prostate cells were infected with a *ras*-carrying virus, no angiogenesis or dysplasia was observed, but when 0.1% of the cells were infected, marked angiogenesis and dysplasia were observed.

In summary, our results suggests that in *ras* transformed MSU-1.1 cells the level of *ras* oncoprotein expression strongly influences transformation *in vitro* and the formation of solid malignant tumors *in vivo*. These findings may provide a rationale for targeting mutant *ras* genes or the *ras* oncoprotein as a potential therapy for cancer because of the possible correlation between *ras* expression levels, angiogenesis and tumorigenicity.

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CHAPTER III

IMMUNOHISTOCHEMICAL DETECTION OF *RAS* P21 PROTEIN IN TUMORS FORMED FROM ±7ß,8∝-DIHYDROXY-9∝,10∝-EPOXY-7,8,9,10 TETRAHYDROBENZO[a]PYRENE (BPDE) TREATMENT OF THE INFINITE LIFESPAN HUMAN FIBROBLAST CELL STRAIN MSU-1.1.

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ABSTRACT

Previous work in this laboratory has shown that a single exposure of MSU-1.1 cells in exponential growth, to(±)-7ß,8∝-dihydroxy-9∝,10∝-epoxy-7,8,9,10-tetrahydrobenzo [a] pyrene (BPDE) caused some of these cells to form foci composed of transformed cells. When the cloned progeny from some of these foci were injected into athymic mice, the injected cells formed benign tumors, low grade spindle sarcomas, fibrosarcomas or round cell sarcomas. Since other studies in this laboratory have shown that MSU-1.1 cells can be malignantly transformed when an activated H-, N-, or v-Ki-*ras* gene is transfected into these cells, we conducted an immunohistochemical study to determine: (1) if the tumors formed by the BPDE-transformed cells exhibit upregulation of *ras* expression, (2) if the tumors expressed a mutant form of the H-*ras* oncoprotein which has a glycine to valine substutition due to a codon 12 point mutation in the H-*ras* gene. The tumors formed by BPDE- transformed cells that formed fibrosarcomas and round cell sarcomas showed a strong positive, immunohistochemical staining with the $p21^{pan\ ras}$ monoclonal antibody. The low grade spindle cell sarcomas had foci of highly malignant round cells ("pearls"), which stained positive with the same $p21^{pan\ ras}$ antibody. The benign tumors were negative. Our results indicate that BPDE-transformed cells that formed fibrosarcoma and round cell sarcoma have a genetic alteration that causes higher expression of the *ras* gene.

INTRODUCTION

Epidemiological studies (1), experiments on tumor induction in animals (2, 3), and *in vitro* studies (4, 5) all indicate that cancer is the result of a multi-step process. Carcinogenic agents (chemical and radiation) damage DNA, and in the process of replication, carcinogen adducts in DNA cause misreplication leading to heritable mutations. This has led to the hypothesis that, in cells some subset of the 100,000 genes when mutated can trigger the aberrant growth characteristic of tumor cells. The critical genetic targets for carcinogens were unknown until the discovery of cellular proto-oncogenes (6-8) and tumor suppressor genes (9). Proto-oncogenes have been shown to play an important role in normal cell growth and differentiation (6). They can be activated to oncogenes by point mutation, gene amplification, deletions, or chromosomal translocation (10). Typical oncogenes that have been identified in human tumors are *ras*, *myc*, and *fos* (10).

Suppressor genes, e.g., retinoblastoma gene (RB), can also play a causal role in carcinogenesis. In this case, it is the failure of the cells to make active protein caused by homozygous loss of DNA sequence, point mutations or large scale deletions in the regulatory sequence of the gene (11). The resulting loss of the normal "suppressor protein" from these cells causes them to have tumor-cell-like characteristics.

There is strong evidence from *in vitro* and *in vivo* studies that the *ras* gene product, p21, plays a significant role in the transformation of many cell types (12, 13). Individual members of the *ras* family of oncogenes H-, K- and N- are found to be "activated" in 25%-40% of human tumors, suggesting that the *ras* gene may be causally involved in these tumors (10).

Many studies have demonstrated that in animals, activation of *ras* genes is one of the many steps involved in transforming normal cells to cells with tumorigenic characteristics (14-16). For example, Sukumar *et al.* (15) reported that in the rat, the alkylating agent

NMU caused mutations in the H-*ras* gene in cells from the mammary gland. The carcinogen also induced tumors in the mammary gland and cells from 80% of the mammary tumors exhibited a mutation in the H-*ras* gene. Similarily, Balmain *et al.* (17) reported that in the mouse, more than 85% of the DMBA induced skin papillomas tumor derived cells had mutations in the H-*ras* gene. Newcomb *et al.* (18) demonstrated that over 75% of thymic lymphomas, induced in mice with radiation or chemical carcinogens caused K-*ras* and N-*ras* mutations. *Ras* mutations have also been identified in lung and hepatic neoplasms of rats and mice after exposure to a variety of carcinogens (3). These findings underscore the high frequency at which *ras* genes are mutated, and strongly suggest that such activated *ras* genes play an important role in carcinogen-induced tumors in animals.

Cell culture techniques have been especially useful in defining the role of the *ras* oncogene in carcinogenesis. Using the DNA transfection technique, several workers have demonstrated the transforming potential of the *ras* oncogenes in human fibroblasts *in vitro* (12, 13, 19). For example, Hurlin *et al.* (12) reported that high expression of the mutated H-*ras* gene can transform a near diploid infinite life-span human fibroblast (MSU-1.1) to a malignant fibroblast, capable of forming rapid growing and progressively invasive tumors in athymic mice. Wilson *et al.* (13) showed that high expression of the N-*ras* oncogene also transformed MSU-1.1 cells to malignant cells. In a similar study, Fry *et al.* (19) demonstrated that the v-K-*ras* gene (with point mutations at codon 12 and 61) expressed at low levels was sufficient to malignantly transform the MSU-1.1 cells. These experiments clearly demonstrate the transforming capabilities of the activated *ras* oncogenes in human cells fibroblasts in culture.

The use of cell culture also offers a method of analyzing human carcinogenesis which is otherwise impossible. However, human cells in culture have been reported to be

refractory to transformation by carcinogens (for review see ref. 20). A major breakthrough involving cell transformation by carcinogens was reported by Yang *et al.* (21), who showed that a single treatment of MSU-1.1 cells with the carcinogen (BPDE) induced foci formation. The cloned focus-derived cells exhibited many of the *in vitro* characteristics of oncogene-transformed cells such as a transformed morphology, anchorage independence and growth in medium lacking exogenous growth factors. These clonally derived cell lines each produced characteristic neoplasms (low grade, and high grade malignant as well as benign) when injected subcutaneously into athymic mice. Since BPDE has been reported to induce mutations in the *ras* gene of human fibroblasts (22), and since high expression of the *ras* oncogene can transform normal fibroblasts to neoplastic fibroblasts, we decided to examine the tumors produced by the BPDE-transformed cells of Yang *et al.* (21) to determine: (1) if the tumor cells exhibited overexpression of the *ras* gene, (2) whether the H-*ras* proto-oncogene has been "activated". These studies were carried out by immunohistochemical staining of tissue sections from tumors with an antibody (Y-13-259) specific for *ras* proteins which would allow us to compare relative expression by differences observed in staining intensity of tumor cells. Additionally, we examined the tumor tissue sections for expression of the specific mutant form of the H-*ras* oncoprotein, that has a glycine to valine substitution, as the result of a mutation in codon 12 of the H-*ras* gene.

Using the immunohistochemical techniques described by Furth *et al.* (23) we found that BPDE-transformed cells that formed high grade malignant tumors (fibrosarcoma and round cell sarcoma) exhibited focally intense to diffuse positive immunoreactivity with the pan *ras* antibody Y-13-259. The low grade malignant spindle cell sarcoma with multi-nodular aggregates of high grade malignant round cells also exhibited positive immunoreactivity with the same monoclonal antibody but only the round cells stained positive, while the other parts of the tumor, as well as the fibromas (benign tumor) stained

negative. None of the tumors exhibited positive immunohistochemical staining to the $p21^{val}$ monoclonal antibody that is specific for the mutant form of the H-*ras* oncoprotein which has a glycine to valine substitution, and is expressed as a result of a point mutation in codon 12 of the H-*ras* gene. These data suggest that BPDE induced a change causing overexpression of the *ras* gene. The increase in *ras* protein expression, however, was not due to the glycine to valine mutation in codon 12 of the H-*ras* gene.

MATERIALS AND METHODS

Tumorigenic cell strains: The development and characteristics of the tumorigenic cell strains which caused the tumors used in this study have been described (12, 21). The v-*sis* transformed MSU-1.1 cell strain that formed the tumors used as a negative control was provided by Dr. Yang.

Immunohistochemistry: This study used formalin fixed, paraffin embedded, tissue sections of tumors induced by MSU-1.1 cells that had been transformed by transfection of the H-*ras* oncogene (positive control), or *sis* oncogene (negative control) or that had been exposed to the chemical agent BPDE. The H-*ras* transformed cells express high levels of a *ras* oncoprotein which has a glycine to valine substitution due to a point mutation in codon 12 of the H-*ras* gene. Sections of tumors approximately 5 μm thick were mounted on poly-l-lysine coated glass slides. Each paraffin section was deparaffinized in xylene, hydrated through decreasing concentrations of ethyl alcohol, and washed in phosphate buffered saline (PBS), pH 7.2. Following each step, the slides were washed in PBS. Endogenous peroxidase activity was inhibited by immersing slides for 10 minutes in absolute methanol containing 30% hydrogen peroxide. Following a 20 minute rinse in PBS, the sections were incubated for 20 minutes with diluted normal goat serum (the species in which the secondary antibody was made) and excess serum was then blotted from them. The sections were incubated with the *ras* specific monoclonal antibodies Y-13-259$^{pan\ ras}$ (Oncogene Science, Inc., Manhasset, NY) which recognizes all *ras* proteins, and NEI-701 p21^{val} which is specific for the H-*ras* protein mutated at codon 12 with a glycine to valine substitution (E.I. Dupont de Nemours,Inc., Cambridge, MA) for 15 minutes with a final working dilution of 1:250. After a 10 minute rinse in PBS, the sections were incubated with a biotinyalated secondary antibody for 30 minutes, and then with the avidin-biotin-peroxidase complex solution for 45 minutes (Vectastain ABC kit, Vector Laboratory, Irvine, CA). The sections were incubated with the chromogen 3,3,

diaminobenzidine (DAB) for 2 minutes. Peroxidase DAB reaction formed a stable brown reaction for the ras p21 protein. Sections were then lightly counterstained with Gill's hematoxylin, dehydrated, cleared, and mounted with non-aqueous resin (Permount; Fischer Scientific, Cincinnnati, OH) observed for staining intensity, and photographed with a microscope.

RESULTS

Tumorigenicity of BPDE-transformed MSU-1.1 cells: Four independent cell strains formed tumors when injected subcutaneously in athymic mice. Details of the tumor-forming capacity and histopathology of these tumors are described in ref. 21. Two strains formed high grade malignant tumors, one a fibrosarcoma and the other a round cell sarcoma. One strain primarily induced a low grade spindle cell sarcoma with focal aggregates of malignant round cell "pearls". These "pearls" formed only a small portion of the tumors, and probably represent a secondary progressive change from low to high grade malignancy. The other cell strain induced fibromas (benign tumor).

Immunohistochemistry: The positive control cell strain tumors, which express the mutant H-*ras* oncoprotein, showed strong positive immunoreactivity (as evidenced by a deep brown cytoplasmic staining) throughout the tumor. Staining was observed in the cytoplasm as well as on the cell membrane. As expected, the degree of positive immunoreactivity observed was similar for both the pan *ras* and the $p21^{val}$ antibody that recognizes the mutant H-*ras* oncoprotein with a glycine valine substitution caused by a point mutation in codon 12 of the H-*ras* gene. The negative control cell strain tumors exhibited no staining with either antibody.

The BPDE-transformed cell strains that formed tumors exhibited different degrees of reactivity with the antibody. The results are summarized in Table 2.1. The round cell sarcoma showed the strongest degree of immunohistochemical staining to the pan *ras* antibody (Figure 3.1). Staining was diffuse and primarily cytoplasmic, with some cells exhibiting membrane staining. The fibrosarcoma had moderately positive immunohistochemical staining in most of the tumor cells, but in some areas cellular staining was pale (Figure 3.1). However, like the round cells just described, a subpopulation in the fibrosarcoma, composed of high grade malignant round cells not

discernible with H&E staining, stained intensely positive with the pan *ras* antibody (Figure 3.1). Within these cells there was cytoplasmic as well as membrane staining. The low grade spindle cell sarcoma stained with the pan *ras* antibody exhibited a positive immunoreactivity only in focal areas containing round cells "pearls" (Figure 3.1). Staining within these cells was intense and mostly cytoplasmic, with a few cells exhibiting membrane staining. Occasionally in these tumors, a small isolated cluster of 5-6 malignant round cells also exhibited strong cytoplasmic immunoreactivity to the pan *ras* antibody (data not shown). The benign tumors were negative.

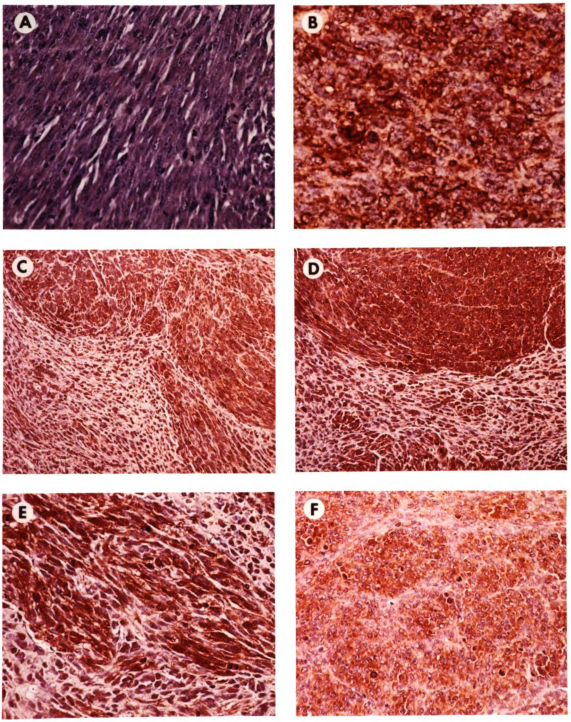
None of the BPDE-transformed cells that formed tumors showed any positive immunoreactivity to the $p21^{val}$ antibody which recognizes the H-*ras* oncoprotein with a glycine valine substitution due to a point mutation in codon 12 of the H-*ras* gene. In order to rule out the possibility that these tumors might be expressing the mutant protein at low levels, we used a panel of tumors formed by H-*ras* $p21^{val}$ cell strains that exhibit low, medium and high expression levels of the H-*ras* $p21^{val}$ oncoprotein. Using the $p21^{val}$ antibody we compared H-*ras* $p21^{val}$ expression in the tumors formed by MSU-1.1 BPDE-transformed cells with the tumors formed by the H-*ras* $p21^{val}$ expressing strains that expresses the same mutant protein at low, medium, and high levels. The results were consistent with those observed earlier, i.e., none of the MSU-1.1 BPDE-transformed cells that induced tumors exhibited any positive immunoreactivity with the $p21^{val}$ antibody that is specific for the glycine valine substitution due to a point mutation in codon 12 of the H-*ras* gene.

Table 3.1 Degree of histochemical staining of representative tumors formed by BPDE-transformed MSU-1.1 cells and stained by *ras* specific antibodies.

Tumorigenic MSU-1.1 derived cell strains	Tumor type	Degree of BPDE transformed tumor cell immunostaining to *ras* antibodies	
		pan *ras*[a]	[b]H-ras[val]
[c]v-*sis*	Low grade spindle cell sarcoma	-	-
[d]H-*ras* 10T	Round cell sarcoma	+++	+++
IC4	Fibroma	-	-
3C1	Low grade spindle cell sarcoma	+[e]	-
2C1	Fibrosarcoma	++	-
4C5	Round cell sarcoma	+++	-

[a]Pan *ras* antibody detects H-, N- and K- *ras* p21 proteins;
[b]H-*ras*[val] antibody is specific for codon 12, H-*ras*, gly->val mutation;
[c]Negative Control
[d]Positive Control
[e]Only focal aggregates of high grade malignant round cells ("pearls") within the tumor stained positive
(-) negative staining; (+) weakly positive; (++) moderately positive; (+++) strongly positive.

Figure. 3.1: Immunohistochemistry for *ras* p21 protein in tumors induced by BPDE-transformed MSU-1.1 cells.(A) Negative control MSU-1.1 v-*sis* tumor (x200), B: H-*ras* 10T tumor,(x200) Positive control. (C) High grade malignant tumor produced by the cell strain 2C1 (x100). Note the strong positive immunoreactivity. (D) Higher magnification (x200) of C. (E) Tumors induced by the cell strain 3C1. Note the intense positive staining in the malignant spindle shaped cells (x200). (F) Strong positive immunoreactivity in this high grade malignant round cell sarcoma (x200) produced by the cell strain 4C5.


A
B
C
D
E
F

DISCUSSION

The present study shows that BPDE-transformed, focus- derived cells induce tumors that exhibit varying degrees of positive immunoreactivity to a pan *ras* antibody that detects H, N, and K *ras* proteins. The high grade malignant tumors had the strongest immunoreactivity to the pan *ras* antibody, indicating that in the process of cellular transformation, exposure to BPDE caused a genetic change that resulted in increased expression of the *ras* proto-oncogene. This was consistent with the studies of Hurlin *et al.* (12) and Wilson *et al.* (13) who reported that MSU-1.1 cells could be transformed by the *ras* gene if it were overexpressed, i.e., markedly upregulated. In their studies only upregulation of the *ras* oncogene (i.e., a suitably mutated gene) was sufficient to cause transformation. The results of the studies reported here indicate that the *ras* gene (s) are overexpressed, but since most mutations at codon 12 or 61 are activating, a negative finding for a single type of mutant protein as we found, leaves open the question of whether another mutation might be present. Additionally, Stevens *et al.* (22) reported that BPDE induced mutations in the H-*ras* gene of human fibroblasts. Our results are consistent with the hypothesis that the genetic alteration induced by BPDE is a mutation in the promoter region of one of the *ras* genes, since this would lead to the increased protein (p21) expression. Another line of evidence to support the immunohistochemical findings comes from the fact that high grade malignant, well vascularized tumors have been associated with increased expression of the H-*ras* oncogene (Louden, *et al.* unpublished studies).

Interestingly, the low grade spindle cell sarcoma "pearls", composed of high grade malignant round cells exhibited strong immunoreactivity to the pan *ras* antibody. We speculate that in these tumors the BPDE-induced genetic alteration was responsible for the induction of the benign phenotype, but the subsequent development of the high grade malignant phenotype involved increased expression of a *ras* gene brought about by a

spontaneous mutation as the cells were being expanded or during the growth of the tumor. This is supported by the fact that the benign tumors formed by BPDE transformed MSU-1.1 cells showed no positive immunoreactivity to the pan *ras* antibody.

From this study one can conclude that the increased expression of the *ras* gene contributes to the development of the malignant phenotype in the BPDE-transformed tumorigenic cells. The data also indicate that the genetic event involved in BPDE induced transformation of MSU-1.1 cells does not involve a codon 12 glycine to valine mutation. Further studies are needed to determine what changes are involved, and which member of the *ras* gene family is responsible for them.

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CHAPTER IV

MORPHOLOGY AND HISTOLOGIC CLASSIFICATION OF TUMORS INDUCED BY TRANSFECTED ONCOGENES OR CARCINOGEN TREATMENT OF THE INFINITE LIFE SPAN HUMAN FIBROBLAST CELL STRAIN MSU-1.1: A MODEL FOR HUMAN SOFT TISSUE TUMORS[1].

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32 pages; 3 Tables; 2 figures

Running head: Tumors induced by oncogenes or carcinogens

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ABSTRACT

A phenotypically normal, near-diploid, infinite life span human fibroblast cell strain, MSU-1.1, developed in this laboratory was treated with ionizing radiation or with the chemical carcinogens (±) -7ß,8∝-dihydroxy-9∝,10∝-epoxy-7,8,9,10-tetrahydrobenzo[a] pyrene (BPDE) or ICR-191, and selected for focus formation. Alternatively, the cell strain was transfected with oncogenes and selected for cells with transformed morphology or focus formation. Cell strains derived from these foci induced tumors in athymic mice. Although all the tumorigenic cell strains were derived from the same original fibroblast cell strain MSU-1.1, the tumors produced by the neoplastically transformed derivatives could be classified into distinct morphological types, having histologic features similar to the spectrum of soft tissue proliferations seen in humans. In traditional pathology terms, the tumors obtained with single phenotypes were fibromas, low grade spindle cell sarcomas, fibrosarcomas, myxosarcomas, malignant fibrous histiocytomas, rhabdomyosarcomas and round cell sarcomas. Most tumors were heterogeneous and exhibited multiple phenotypes of those described. In some cases the type of tumor formed was specific to the type of transforming agent used.

INTRODUCTION

Epidemiological studies indicate that the development of human cancer is a multi-step process,[1-3] and involves exposure to carcinogens in the environment.[4] Carcinogens that act as mutagens mediate the steps leading to malignant transformation *in vitro* [5-8] and *in vivo*.[9-11] The targets for cellular transformation were unknown until the discovery of oncogenes and their normal cellular counterparts, proto-oncogenes.[12,13] Evidence indicates that the conversion of proto-oncogenes to oncogenes in a variety of human and animal tumors results from the mutagenic action of carcinogens.[12-15] Considerable evidence has also emerged supporting the existence of a second class of cancer causing genes, called tumor suppressor genes, e.g., the retinoblastoma gene (Rb) and the p53 gene. The loss or inactivation of suppressor genes can play a causal role in tumor development.[16] It is now assumed that neoplastic transformation results when a single cell has acquired appropriate changes in some combination of proto-oncogene and/or suppressor genes, although no one has yet successfully identified the required changes in any type of human cell.[17]

Many of the insights regarding the mechanisms of cell transformation were developed from analyzing cells grown in culture.[13,14] Of particular value has been the use of DNA transfection, which allows one to place activated oncogenes into cells. The phenotypic change caused by the expression of such genes can then be determined.[13,14,18] The procedure is also used to return suppressor genes to tumor cells that have lost them, to determine what property they confer.[19,20] Most of the early research on transformation of cells in culture utilized an immortalized mouse fibroblast cell strain, NIH 3T3.[13,14,18] Transfection with the *ras* oncogene induced morphologically transformed 3T3 cells that induced tumors in athymic mice.[21,22]

Similar experiments have also been conducted using other infinite life span rodent fibroblast cell lines.[23-26] The extension of such transformation studies to human cells in culture has been limited by the lack of human cells immortalized *in vitro* but essentially normal in other characteristics. Studies of the ability of transfected oncogenes to transform human cells into neoplastic cells have been carried out with tumor-derived human cells that are no longer tumorigenic [27] or with human cells immortalized by Simian virus 40,[28] but such target cells already possess many of the characteristics of tumorigenic cells and so are not so useful for studying the steps or changes involved in human carcinogenesis.

Soft tissue sarcomas account for approximately 1% of human malignancies and approximately 2% of cancer deaths.[29] These tumors of mesenchymal origin comprise a heterogeneous group of neoplasms characterized morphologically by spindle cells. The cells of origin of soft tissue tumors are presumed to be fibroblast, lipoblast, myoblast, osteoblast and endothelial cells, but rigorous demonstration of this is lacking since unique biochemical markers for cells such as fibroblasts have not been described. There is evidence that specific types of oncogene activation have occurred in various human fibrosarcoma-derived cell lines, suggesting that such changes play a causal role in the transformation of these cells. For example, the *N-ras* oncogene has been identified in human fibrosarcoma derived cell lines [30,31] and shown to play a causal role in malignant transformation of these cells.[32] Since we now know that oncogenes such as *myc* and *ras* are activated in tumors of mesodermal and epithelial origin,[16] investigations of the genetic and molecular mechanisms underlying tumorigenesis in fibroblasts should also aid our understanding of the genesis and biology of carcinomas. Recently, workers in this laboratory developed a human fibroblast cell strain, MSU-1.1,[33] which has a stable, near-diploid karyotype and is non-tumorigenic. Using this cell strain, transfection studies with

H-, *N-* and *v-K-ras* oncogenes were carried out in which MSU-1.1 cells were converted to cells that grew as a focus or formed large-sized colonies in 0.33 % agarose. The progeny of oncogene-transformed fibroblasts isolated from foci or agarose colonies produced progressively growing and invasive malignant tumors in athymic mice.[34-36] When MSU-1.1 cells were transfected with a *v-sis* oncogene, the transformed cells produced benign tumors in athymic mice.[37] Treatment of MSU-1.1 cells with carcinogens also induced focus formation by these cells. The progeny of the focus derived cells exhibited many of the *in vitro* characteristics of the oncogene-transformed cells and were also able to form tumors in athymic mice. In this manucsript we characterize the tumors formed by the tumorigenic MSU-1.1 cell strains. In addition, we studied tumors formed by re-injection of cells derived from primary tumors to determine the consistency and/or reproducibility of the primary tumor phenotype. We also examined the relationship between the tumor histology, grade of malignancy, and the expression of different oncogenes. In characterizing the tumors, we found that most of the them exhibited multiple phenotypes i.e., a mixed population of tumor cells, while other tumors exhibited a single phenotype. We identified seven distinct morphologic types. The histological features exhibited by these tumor cells were very similar to those observed in humans.

MATERIALS AND METHODS

Tumorigenic cell strains:

All tumors examined in this study were generated from the transfection and/or carcinogen treatment of MSU-1.1 cells. The origin of these independent cell strains are described in Table 4.1 along with references to the papers describing the original data.

Tumorigenicity:

Exponentially growing cells were injected subcutaneously into the shoulder and/or flank of 4-6wk old athymic Balb/C mice and the tumors were measured weekly with a vernier caliper. After a minimum of 4 weeks of growth most tumors were removed and portions returned to culture to establish tumor-derived cell lines. Other sections were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned at 6-8μm and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), or alcian blue if necessary and examined by light microscopy.

Histologic examination of tumors:

The tissue slides were first examined without regard to the treatment the cells had received in order to determine if tumors were present, and if so, to group the tumors according to the pattern of neoplastic growth exhibited by the tumor cells. After this examination, the tumor slides were evaluated again to characterize and identify tumors whose cells exhibited single versus multiple phenotypes. Tumors were described using traditional pathologic terms used to describe soft tissue tumors in humans.[39] Tumors that exhibited abundant myxoid stroma were stained with periodic acid-Schiff (PAS) or alcian blue to determine if this matrix was composed of mucopolysaccharides.

Table 4.1. Relation of tumor size and rate of growth to the transforming agent used with MSU-1.1 cells

Transforming agent	No. of independent Cell strains	Growth rate of tumor[a]	Average size of tumor[b]	Reference
H-*ras*[c]	09	Rapid	< 1.0 cm	34[d]
H-*ras*[e]	01	Slow	≥ 0.5 cm	d
N-*ras*	06	Rapid	< 1.0 cm	35
V-H-*ras*	01	Rapid	< 1.0 cm	f
V-K-*ras*	05	Rapid	< 1.0 cm	36
K-*ras*	01	Rapid	< 1.0 cm	f
V-*sis*	04	Slow	≥ 0.5 cm	37
Carcinogen Treatment				
BPDE	05	Rapid	< 1.0 cm	7
ICR-91	06	Slow	>0.5 cm	f
γRadiation	06	Rapid	<1.0 cm	38

[a]Rapid growth: Tumor attained a diameter of 1.0 cm or greater in 30 days; Slow growth: Tumor attained a diameter of 0.5cms or less in 30 days. [b]Average size of tumor when removed at necropsy. [c]Ras p21 protein is expressed 3-7 times above parental MSU-1.1 cells[d]. Four of these are described in ref.30; additional strains were derived for this present study [e]Ras p21 protein is expressed 1-2 times above parental MSU-1.1 cells [f] McCormick, JJ (unpublished studies)[g] Reinhold, D., *et al.*

RESULTS

Carcinogen treatment and/or oncogene transfection of MSU-1.1 cells yielded 44 independently transformed cell strains, isolated from foci or large-sized colonies growing in soft agarose. These transformed cells induced 242 primary tumors in athymic mice.

Comparative growth rate, size and latency of primary tumors induced by transfection of oncogenes or carcinogen treatment of MSU-1.1 cells:

After reviewing the data which describes the latency, size and rate of growth of all tumors examined, marked differences among tumors were observed. To understand these relationships comparative analyses among tumors induced by transfected oncogenes or carcinogen transformed cells were made. These data are summarized in Table 4.1.

Tumors formed by oncogene transfected cells:

MSU-1.1 *ras* oncogene-transformed cells induced rapidly growing subcutaneous nodules, greater than 1 cm in diameter that were well vascularized and friable. The tumors formed by the *ras* oncogenes transformed cells had a short latency period except the tumors formed by H-*ras* transformed cells that express the *ras* oncoprotein at low levels. The *v-sis* oncogene transformed MSU-1.1 cells formed slowly growing tumors that averaged approximately 0.5 cm in diameter after 4-6 weeks at which time most of these tumors exhibited a marked decrease in rate of growth.[37] The tumors were firm and quite hard, with no visible blood vessels.

Tumors formed by chemical carcinogen transformed cells:

Cells in the MSU-1.1 lineage that were transformed into focus forming cells by treatment with the carcinogens BPDE, or ICR-191 induced tumors of various sizes. The majority of malignant tumors grew rapidly with a short latency, were soft and friable and were well

vascularized. The benign tumors were small and grew at a slower rate than the malignant tumors. Some of the benign tumors never reached more than 0.5 cm in diameter. The tumors formed by MSU-1.1 cells transformed by the carcinogen ICR-191, grew more slowly than did the tumors induced by BPDE-transformed cells.

Tumors formed by ionizing radiation-transformed cells:

Most of the ionizing radiation-transformed cell strains induced rapidly growing tumors. However, a few independent cell strains induced small, slow-growing tumors, some of which regressed after 2-3 weeks of growth.

Comparative growth rate, size and latency of secondary tumors induced by transfection of oncogenes or carcinogen-treatment of MSU-1.1 cells:

The re-injection of primary tumor-derived cells formed tumors that are referred to as secondary tumors. In almost all cases the secondary tumors were larger, and had a shorter latency than the primary tumors. This was true whether the primary-transformed MSU-1.1 cells were derived from carcinogen treatment or oncogene transfection.

Histopathology of tumors induced by oncogene transfection and/or carcinogen treatment of MSU-1.1 cells:

Multiple, independent, clonally-isolated transformed cell strains were selected after oncogene transfection, carcinogen treatment and re-injection of primary, tumor-derived cells. This gave us 44 primary tumorigenic cell strains and 34 tumor-derived (secondary), tumorigenic cell strains derived from MSU-1.1 cells. Since all strains were injected into multiple animals, we had 242 primary tumors and 76 secondary tumors for analysis. Our detailed examination identified tumors with single phenotypes, tumors with biphasic pattern, tumors with "pearls" and tumors with a heterogeneous blend of multiple phenotypes. Each tumor type was differentiated based on a defined histological criteria.

Tumors with single pattern:

In these tumors, the tumor cells exhibited a single phenotype and were classified according to the traditional nomenclature used to describe soft tissues tumors.

Fibroma:

These tumors consisted of a discrete, spindle, and stellate-shaped fibroblasts widely separated by hyalinized collagen stroma as shown in Figure 4.1, A and B. These cells had a low nuclear size to cytoplasmic ratio, elongated nuclei with condensed chromatin and no mitotic figures (40x objective) were observed. The tumor cells had scant bipolar cytoplasm which was not easily discernible.

Low grade spindle cell sarcoma:

These tumors were discrete with moderate cellularity, consisting of a monomorphic proliferation of well-differentiated, spindle-shaped fibroblasts, arranged loosely in a collagenous stroma which varied from fine to coarse (Figure 4.1, C and D). These tumor cells had a low nuclear to cytoplasmic size ratio with at least 2 distinct nucleoli, condensed chromatin and at least 1 mitotic figure per high power (40x objective) field. Tumor cell cytoplasm was indistinct, bipolar and scant as the nucleus filled most of the cytoplasm.

Myxosarcoma:

This tumor type was characterized by a large, nodular, highly cellular, subcutaneous mass with an abundant myxoid matrix, very little collagen, and spindle-shape cells that varied in shape and size. The cells were arranged in irregular bundles. Poorly defined fascicles were accompanied by a rich intervening matrix of mucopolysaccharides with a loose textured feathery pattern that stained readily with alcian blue or periodic acid-Schiff (PAS). An abundant myxoid matrix was present throughout the tumor mass (Figure 4.1, E and F).

The tumor cells had low nuclear to cytoplasmic size ratio with some cells having 2-3 distinct nucleoli with moderate hypochromasia, reticular chromatin and 1-3 mitotic figures

per high power (40x objective) field. The cytoplasm of these tumors cells were not discernible.

Malignant fibrous histiocytoma:

The tumors in this category formed large, subcutaneous masses with a high degree of cellularity and small broad interweaving bands of mature and immature, fibrillar collagenous stroma. Most of the cells were arranged in short irregular bundles (Figure 4.1, G and H), accompanied by a loose to dense meshwork of collagen fibers. The tumor cells were plump, oval to spindle-shape, and had moderate cellular atypia. Some unusually large, round "histocytic like" cells were located randomly within the tumors. These tumor cells had a variable nuclear cytoplasmic size ratio with some cells having as many as 5 nucleoli, moderate hypochromasia, reticular chromatin and 3-5 mitotic figures per high power (40x objective) field. There was marked cytoplasmic variability among tumor cells.

Fibrosarcoma:

The tumors with this pattern were large, nodular to multinodular, highly infiltrative subcutaneous masses that distorted the architecture of the surrounding tissue. They consisted of highly cellular, dense sheets of spindle-shaped cells closely resembling confluent fibroblasts in tissue culture (Figure 4.2, A and B). The tumor cells were tightly packed and arranged in interdigitating and interweaving fascicles (herringbone pattern), with variably sized bundles and some gentle whorls with no intercellular collagenous stroma. The tumor cells had a very low nuclear to cytoplasmic size ratio with several cells having 5 or more large prominent nucleoli, marked nuclear hypochromasia, reticular chromatin, and 6-8 mitotic figures per high power (40x objective) field. Cell cytoplasm was variable and some tumor cells had moderate cytoplasmic vacuolation.

Rhabdomyosarcoma:

The tumors with this pattern were large, solid, highly infiltrative, subcutaneous masses that disrupted the architecture of the normal subcutaneous tissue. These tumors consisted of an extensive proliferation of a pleomorphic population of large, round-to-ovoid and spindle-shaped cells, and many multinucleated cells (Figure 4.2, C and D). The cells were densely packed in an indistinct, mature collagen stroma. The tumor cells had pronounced variability in cell shape, cell size, nuclear characteristics and nuclear to cytoplasmic size ratio, with as many as 4 nucleoli in some cells and 3-5 mitotic figures per high power (40x objective) field. Over 70% of the tumor cells had abundant, basophilic, foamy-to-vacuolated and sometimes eosinophilic cytoplasm with a "strap like" appearance suggestive of rhabdomyoblasts.

Round cell sarcoma:

This tumor type was characterized by a highly cellular, dense sheet of large, round-to-oval cells with no intervening stroma (Figure 4.2, E and F). The tumor cells had a very low nuclear to cytoplasmic size ratio, 3-5 large nucleoli and 6-8 mitotic figures per high (40x objective) power field. The tumor cells had distinct cell borders and scant basophilic cytoplasm with fine vacuoles. Most of the tumor cells had marked atypia and a round nucleus. The nuclei of several tumor cells appeared convoluted, with marked variability and were often vacuolated, with a fine reticular chromatin pattern, hypochromasia and a marginated chromatin.

Figure 4.1. Photomicrograph of tumors with a single phenotype. A: fibroma, characterized by abundant collagenous matrix (H&E, x100). B: well-differentiated fibroblasts observed in fibromas (H&E, x180). C: Low-grade spindle cell tumor, with fibrillar collagen (H&E, x100). D: Note the prominent nucleoli in the low-grade malignant cells (H&E, x180). E: Myxosarcoma with no apparent collagen (H&E, x100) F: Myxomatous matrix (H&E, x180). G: Malignant fibrous histiocytoma "like", with irregularily arranged collagen (H&E,x100). H: Note "epitheliod like" cells in malignant fibrous histiocytoma(H&E, x180).

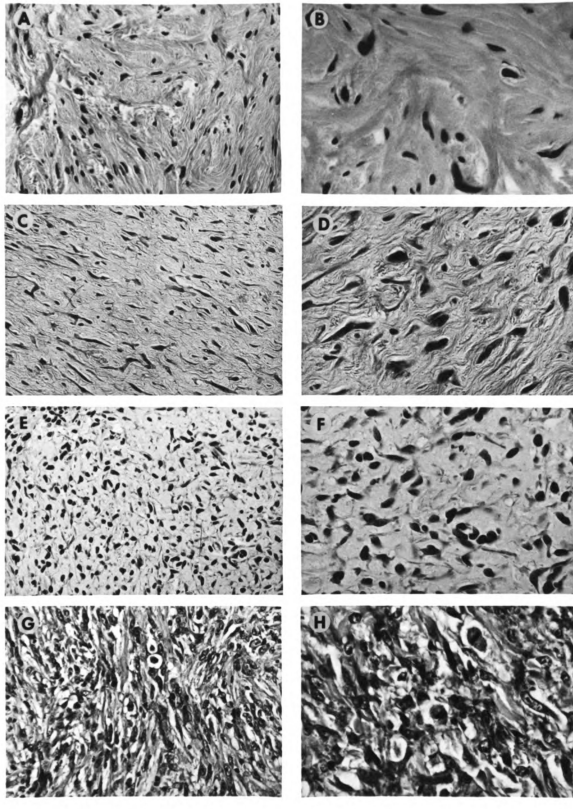
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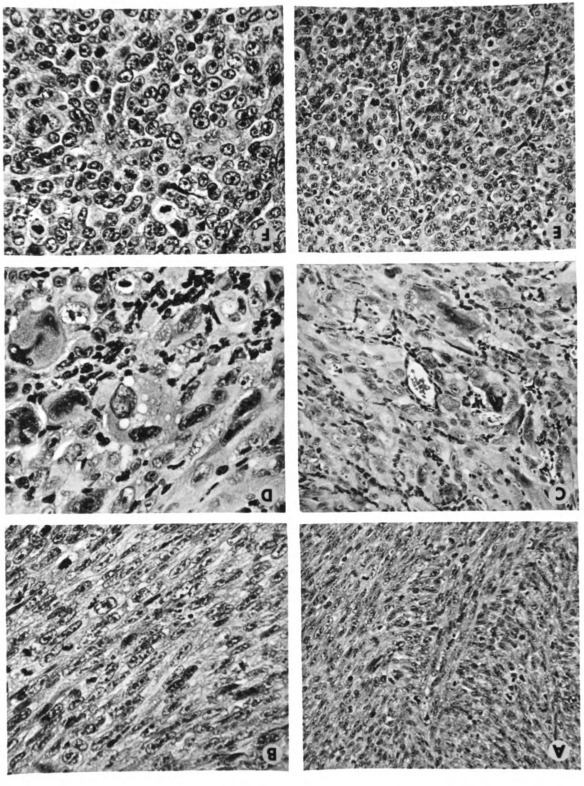
Figure 4.2. Photomicrograph of high grade malignant tumors with a single phenotype. A: fibrosarcoma exhibiting "herring bone" pattern (H&E, x100). B: anaplastic fibrosarcoma cells with prominent nucleoli (H&E x180). C: Rhabdomyosarcoma "like" with "strap cells". D: Large "strap cells" (H&E, x180). E: Monomorphic population of round cells in round cell sarcoma (H&E, x100). F: Anaplastic tumor cells with mitotic figures in a round cell sarcoma (H&E, X180).


A
B
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Tumors with bi-phasic pattern:

These tumors exhibited two distinct cellular phenotypes that were well demarcated by a narrow zone of dense fibrous connective tissue. The tumor cells in each separate zone were monomorphic, with no evidence of an overlap or mixing of the cell populations between the different zones.

Tumors with pearls:

These tumors were classified as such because of the presence of multiple variably sized discrete aggregates of a monomorphic population of anaplastic round cells. The "pearls" were randomly distributed, and often caused compression of the adjacent less anaplastic spindle shape tumor cells. The round shaped tumor cells in the pearls had similar histologic features to the tumor cells in the round cell sarcoma.

Heterogenous blend of multiple patterns:

Typically, these tumors exhibited several different patterns in a haphazard manner. Each phenotype identified in these tumors was clearly discernible, but in some instances there was considerable mixing of tumor cells with different phenotypes.

Correlation of tumor type with inducing oncogene and/or carcinogen:

We examined the tumors formed by carcinogen or oncogene transformed cells to determine if a particular tumor phenotype was influenced by carcinogen treatment or the expression of certain transfected oncogenes. The results are summarized in Table 4.2, which shows the relationship between tumor types and the different transforming agents used with MSU-1.1 cells.

Tumors formed by *H-*, *K-* or *N-Ras* oncogene transformed cells:

Most of these tumors were highly malignant and single pattern tumors were classified as rhabdomyosarcoma, fibrosarcoma and round cell sarcoma. The tumor cells in

Table 4.2 Relation of the transforming agents to primary tumors with unique histologic patterns[a]

Transforming agents	Independent cell strains	No. of Primary tumors examined [b]	Single histologic pattern[c]	Biphasic histologic pattern[d]	Aggregates of round cells "pearls"[e]	Heterogenous blend of "multiple histologic patterns"[f]
H-Ras[g]	09	49	5 (5) 7 (16)	6,7 (13) 5,7 (8)	2,7 (1) 4,5,7 (5)	2,3,4,5,6,7 (1)
H-Ras[h]	01	09	2 (7)	-	-	2,5,6,7 (2)
N-Ras	06	32	6 (4)	5,7 (9)	-	5,6,7 (19)
v-H-Ras	01	02	-	5,6 (1)	-	3,5,6 (1)
v-K-Ras	05	09	3 (2)	-	-	3,4,5,7 (2) 3,5,7 (5)
K-Ras	01	01	5 (1)	-	-	-
v-Sis	04	65	1 (5) 2 (4) 4 (2)	2,4 (3) 1,2 (1)	1,2,7 (16)	1,2,3,4,5 (28) 2,3,4,5,6 (6)
Carcinogen Treatment						
BPDE	05	27	4 (1) 5 (2) 7 (4)	1,2 (2)	1,2,5,7 (3)	2,4,5,7 (3) 1,2,3,5,7 (4) 1,2,3,4,5,6,7 (8)
ICR-191	06	08	1 (1)	1,2 (4)	1,7 (1)	1,2,5 (2)
γRadiation	06	39	2 (5)	2,5 (1)	5,7 (8)	2,5,7 (25)

No. of tumors in parenthesis (); [a]1- Fibroma; 2- Low grade spindle cell sarcoma; 3- Myxosarcoma; 4- Malignant fibrous histocytoma; 5- Fibrosarcoma; 6- Rhabdomyosarcoma; 7- Round cell sarcoma.; [b]Tumors formed from injection of primary transformed cells; [c]Single pattern: tumors had cells of one phenotype; [d]Biphasic pattern: tumors had cells of 2 phenotypes clearly demarcated by fibrous band; [e]Pearls: variably size, nodular, discrete aggregates of anaplastic round cells which compressed surrounding tumor cells; [f]Heterogenous blend: tumors composed of cells with different phenotypes haphazardly arranged. [g]*Ras* p21 protein expressed 3-7 times above parental MSU-1.1 cells; [h]*Ras* p21 protein expressed 1-2 times above parental MSU-1.1 cells.

heterogeneous tumors, exhibited various combinations of these patterns and in a few tumors well differentiated spindle shaped tumor cells were present. Large, prominent, ectatic and thrombosed blood vessels were present in most tumors induced by *ras* transformed cells. In some tumors, the vascular pattern was sinusoidal. In a few primary tumors, tumor cells were present in blood vessels but distant metastasis was not observed. However, the rapid growth of the primary tumors would probably not allowed us to observe the metastasis at distant sites. Another feature of the tumors induced by cells transformed by this oncogene, was necrosis. Coagulative and individual cell necrosis were prominent with varying degrees of inflammation typified by abundant neutrophils and lesser numbers of macrophages, lymphocytes, and plasma cells.

One *ras*-transformed cell strain that expressed the *H-ras* oncogene at low levels consistently induced low grade spindle cell tumors. The *K-ras* transformed cell strain that expressed the *ras* oncogene product at high levels, was also consistent in inducing fibrosarcoma. However, more *H-ras* and *K-ras* transformed cell strains are needed to confirm these observations.

The phenotypes exhibited by tumor cells in the secondary tumors were similar to the phenotypes observed in the primary tumors (Table 4.3). However, while in some of the primary tumors non-malignant cells were observed, in the secondary tumors only highly malignant tumor cells were present.

Tumors formed by *v-H-ras* and *v-K-ras* oncogene-transformed cells:

v-H-ras and *v-K-ras* transformed MSU-1.1 cells express these oncogenes at low levels. Some *v-K-ras* tumors were classified as myxosarcomas while other tumors exhibited a mixed pattern. Individual cell necrosis was evident in some tumors with multiple phenotypes. The myxosarcomas were not as well vascularized as other tumors induced by other *ras* genes.

Table 4.3 Relation of the transforming agents to secondary tumors with unique histologic patterns[a]

Transforming agents	Independent cell strains	No. of secondary[b] tumors examined	Single histologic pattern[c]	Biphasic histologic pattern[d]	Aggregates of round cells "pearls"[e]	Heterogenous blend of "multiple histologic patterns"[f]
H-Ras	06	14	-	5,7 (9)	4,5,7 (5)	-
N-Ras	06	12	-	5,7 (5)	-	5,6,7 (7)
v-H-Ras	01	05	-	5,7 (5)	-	-
v-K-Ras	05	06	-	-	5,7 (2)	3,4,5,7 (4)
K-Ras	01	04	-	-	5,7 (4)	-
v-Sis	04	16	-	1,2 (2)	2,4,7 (10)	4,5,6,7 (4)
CARCINOGEN TREATMENT						
BPDE	05	12	-	2,6 (4)	5,7 (4)	2,4,5,7 (4)
γRadiation	06	07	-	-	5,7 (7)	-

[a] 1-Fibroma; 2- Low grade spindle cell sarcoma; 3- Myxosarcoma; 4- Malignant fibrous histocytoma; 5- Fibrosarcoma; 6- Rhabdomyosarcoma; 7- Round cell sarcoma. [b]Secondary tumors: tumors formed from re-injection of primary tumor derived cells; [c]Single pattern: tumors had cells of one phenotype; [d]Biphasic pattern: tumors had cells of 2 phenotypes clearly demarcated by fibrous band; [e]Pearls: variably size, nodular, discrete aggregates of anaplastic round cells which compressed surrounding tumor cells; [f]Heterogenous blend: tumors composed cells with different phenotypes haphazardly arranged.

v-H-Ras transformed MSU-1.1 cells induced tumors with different patterns (Table 4.2). The different phenotypes in these mixed pattern tumors were similar morphologically to the tumor cells in single pattern tumors. The *v-H-* and *v-K-ras* transformed cells that formed secondary tumors exhibited a more malignant phenotype than did the tumor cells in the primary tumors.

Tumors formed by the *v-sis* oncogene:

The single pattern tumors induced by cells transformed by this oncogene were classified as fibromas, low grade malignant spindle cell sarcomas and malignant fibrous histiocytomas (Table 4.2). Also, as was observed with other transforming agents, several tumors exhibited a heterogeneous blend of phenotypes. The fibromas induced by this oncogene were generally composed of well differentiated fibroblasts, with abundant to moderate amounts of collagen and a low mitotic activity. Some tumors with a long latency exhibited a characteristic biphasic pattern, while others had focal aggregates of anaplastic round cells with a high mitotic index ("pearls"). In a few cases the well differentiated cells were interspersed with the more anaplastic cells. In these cases there was no clear demarcation between morphologically benign and malignant tumor cells. In contrast to the tumors induced by MSU-1.1 cells transformed by the *ras* oncogene, necrosis was not a prominent feature of these tumors.

The tumor cells in the secondary tumors exhibited benign as well as the malignant phenotype, but for the most part the phenotypes exhibited by the primary and secondary tumors were similar.

Tumors formed by carcinogen-transformed cells:

Treatment of MSU-1.1 cells with the carcinogens BPDE, ICR-191 and ionizing radiation induced focus forming cells. When injected into athymic mice, the cloned progeny from these foci induced a variety of tumors that were classified as fibroma, low grade malignant spindle cell sarcoma, malignant fibrous histiocytoma, fibrosarcoma, and round cell

sarcoma. BPDE was the only carcinogen studied that yielded transformed cells that induced tumors classified as malignant fibrous histiocytoma. The carcinogen-transformed cells induced tumors with similar histologic features to the tumors induced by cells transformed by *ras* and *sis* oncogenes. Immunohistochemical analysis demonstrated that the cells comprising "pearls", fibrosarcoma and round cell sarcoma induced by BPDE-transformed cells, showed a stronger positive immunoreactivity when stained with a monoclonal pan *ras* antibody that detects the H-, K- or N- *ras* family of proteins (data not shown), than did the surrounding tissue in the other kinds of tumors produced by these transformed cell strains. However, these tumor cells did not stain when we used an antibody that detects the mutant protein $p21^{val}$, which has a point mutation in codon 12 of the *H-ras* gene (C. Louden, unpublished studies).

The ICR-191 transformed cells induced tumors with malignant and benign phenotypes. Some ICR-191 transformed cells formed slow-growing tumors which sometimes regressed. Those that did not regress exhibited abundant collagenous stroma with low cellularity and were classified as benign to low grade malignant tumors. The regressing tumors were similar histologically, but in addition there was evidence of severe inflammation. Ionizing radiation transformed MSU-1.1 cells induced malignant tumors with different degrees of differentiation.

Neovascularization (angiogenesis) of MSU-1.1 derived tumors:

The degree of neovascularization in all tumors was determined by visual examination for the number of blood vessels seen with the 20x objective. The presence of endothelial cells with red blood cells in the lumen was used to define blood vessels. The degree of vascularity was characterized as low, moderate, or high, depending on the number of vessels seen. All the fibromas, regardless of the source of the tumorigenic MSU-1.1 cells that produced them had exhibited a low degree of vascularization. Similarly, all the low

grade malignant tumors showed a moderate degree of vascularization while the high grade malignant tumors were very well vascularized (data not shown).

Discussion

In this study we report that when MSU-1.1, an infinite life span human fibroblast, is transfected with different oncogenes or treated with carcinogens, the transformed cells form connective tissue tumors with various morphologic features. The morphologic features of these tumor cells vary from well-differentiated (stellate to fibroblastic) to undifferentiated (round cells). The tumors have histological features similar to the various soft tissue proliferations seen in humans. We also show that oncogene and/or carcinogen transformed MSU-1.1 cells, form tumors with a specific pattern. For example, the *v-sis* oncogene and the carcinogen ICR-191, transformed MSU-1.1 cells formed tumors composed of non-invasive, well differentiated fibroblasts with abundant collagen. In contrast, *ras* oncogene transformed MSU-1.1 cells formed tumors composed of poorly differentiated cells which invade surrounding tissue. Studies by Yang *et al*[37] indicated that the *v-sis* oncogene, or increased expression of its cellular homologue, platelet derived growth factor B, may play a causal role in the induction of well-differentiated fibroblastic tumors. To further examine the relationship between PDGF(B) expression and tumor type, Yang *et al*[37] analyzed BPDE-transformed MSU-1.1 cells for increased expression of PDGF(B) as these cells grew in cell culture medium lacking exogenous growth factors. The results showed that one cell strain that consistently induced well differentiated fibroblastic tumors, expressed an increased level of PDGF(B) mRNA similar to that found in the human fibrosarcoma cell line HT1080. In this case it is possible that PDGF (B) promoter is mutated by BPDE in these cells causing unregulated synthesis.[7] The well-differentiated fibroblastic tumors may have a genetic change in the *sis* gene. PDGF(B) is presumed to exert its proliferative effect in neoplasia and desmoplasia through autocrine and/or paracrine interactions as normal mesenchymal cells do not synthesize PDGF but

respond to it.[40-43] Palman *et al.*,[44] studied the relationship between PDGF(B) and PDGF-R(B) expression in soft tissue tumors. They reported that a large number of benign and malignant soft tissue tumors expressed PDGF(B) while as expected almost all tumors expressed PDGF-R(B). These data support earlier reports that the expression of PDGF(B) and/or its receptor plays a significant role in the development of soft tissue tumors.[40-43] Our data are in agreement with those findings and of others, who reported that PDGF(B), and *ras* proto-oncogenes were activated in fibrosarcoma as well as other human soft tissue tumors.[30-31,45] Maillet and Robinson[45] reported that *H-* and *N- ras*, *sis* and *c-myc*, oncogenes were activated in a variety of human soft tissue tumors that they examined. However, the relationship between the oncogenes expressed and the tumor type was not determined. From the results of that study, they concluded that these oncogenes play a causal role in the etiology of these tumors in humans. The results of our study are in agreement with those findings, but in addition, we show that the different oncogenes may determine the tumor phenotype.

Based on the biological behavior and the grading system of Costa *et al.*,[46] Trojani *et al.*,[47] and Potter *et al.*,[48] the tumors with single phenotypes types observed can be classified as benign (fibroma), low grade malignant (spindle cell sarcoma and myxosarcoma), and high grade malignant (malignant fibrous histiocytoma, fibrosarcoma, rhabdomyosarcoma and round cell sarcoma.

ICR-191 induced primarily benign and low grade malignant tumors. This carcinogen causes frameshifts mutations,[49-51] rather than point mutations. Frameshift mutations in structural genes are more likely to result in the loss of production of a functional protein than activation of a proto-oncogene. If this is the case we expect to find loss of expression of a suppressor gene. Studies to identify such genes are currently in progress. BPDE and ionizing radiation-transformed MSU-1.1 cells induced low and high grade malignant tumors and benign tumors as well. BPDE causes mainly point mutations,[18]

whereas ionizing radiation causes point mutations as well as deletions.[16] Deletions or mutations in cell DNA could inactivate tumor suppressor genes and/or activate dominant acting proto-oncogenes which can play a role in tumor development. Suppressor gene activity is now under study in these cells.

Most *ras* transfected cells induced high grade malignant tumors. In addition, the intraperitoneal (i.p.), intracardiac (i.c.), and tail vein injection of athymic mice with *H-* and *N-ras* tumor-derived cells induced multiple, various sized, tumor cell colonies in the lungs, liver, kidneys, testicles, heart and occasionally in the brain. The ability of other oncogenes and/or carcinogen transformed cells to induce experimental metastatic tumor colonization in various organs has not yet been tested. Since most of the *ras* oncogene-transformed cells induced highly malignant tumors it was surprising that one *H-ras* transfected cell strain that expresses the oncoprotein at low levels, consistently induced well differentiated fibroblastic tumor (C. Louden, unpublished studies). Additional tumorigenic MSU-1.1 cells that express the *H-ras* oncoprotein at low levels will be studied to better characterize this relationship. Of major interest are the number of tumors with histological features of rhabdomyosarcoma, a striated muscle tumor. Interestingly, mutations in the *H-ras* oncogene have been reported in human rhabdomyosarcomas;[52] these data suggest that non-*H-ras* and carcinogen transformed cells that form these types of tumors should be examined for altered expression of this *ras* gene product. Immunocytochemical characterization for tumor specific markers such as Myo-D which identifies rhabdomyosarcoma[53] are being undertaken to determine if such tumor cells express this muscle tumor specific marker. The *v-K-ras* oncogene induced a large number of myxoid tumors. Our search of the literature did not reveal any reported association between *v-K-ras* and myxoid neoplasms and to our knowledge this is the first reported observation.

Round cell sarcomas were induced by the *ras* oncogenes as well as carcinogens. The carcinogens we studied are mutagens and have been reported to cause activating mutations in the *H-ras* proto-oncogene.[5,18]

In many cell types including fibroblasts the *ras* protein p21, plays a critical role in growth and differentiation.[54] Recent evidence indicates that binding and subsequent phosphorylation of receptor tyrosine kinases such as, PDGF or EGF, increases the rate of *ras*, guanine nucleotide exchange and this increases the proportion of bound GTP.*ras*.[55-58] The increase in the proportion of GTP-bound *ras* activates MAP kinases with subsequent increased transcriptional activity of genes such as *Raf-1*, *fos* and *jun*, leading to increased cell proliferation.[60-61]

All the high grade malignant tumors were well-vascularized, and in some cases large dilated blood vessels were observed on gross examination of some tumors. These data are in agreement with the reports of others,[62,63] who found that, the ability to stimulate neovascularization is necessary for the development of solid malignant tumors. Based on our observations, the degree of vascularization in tumors correlates with the degree of malignancy.

As reported by others [29,39,64] and observed in our study, the tumors displayed single and/or multiple histologic patterns. While various combination of patterns were observed in heterogeneous tumors and biphasic tumors, tumors with "pearls" exhibited only certain combinations. For example in the primary tumors, myxoid tumors induced by *v-K-ras* transformed cells, did not have a tendency to form "pearls" while *v-sis* transformed cells that induced tumors did. However, in the secondary tumors induced by *v-K-ras* oncogene transformed cells "pearls" were noted. We attribute the presence of "pearls" and other high grade malignant phenotypes observed in combination with well differentiated tumor cells, to an additional genetic event that caused progression from the benign phenotype to

the malignant phenotype. Brooks [64] suggests that the presence of the second pattern in soft tissue tumors may represent the phenomenon of "antigenic shift" which is linked to mesenchymal differentiation. It is clear from this study, that the parental cell strain MSU-1.1, derived from a diploid fibroblast, does not have a "fixed" fibroblast phenotype, since it gives rise to several tumors with different phenotypes. MSU-1.1 cells carry a characteristic drug resistance marker which allows selection of the MSU-1.1 derived cells when the tumor cells are placed in culture. This assures us that the tumor cells are derived from cells in the MSU-1.1 lineage and are not of mouse origin. Additional evidence that the tumors were formed by MSU-1.1 derived cells is that, in those cases in which we have karyotyped the tumor-derived cells, they carry the two distinctive marker chromosomes of the MSU-1.1 cell strain.

As these studies make clear, the MSU-1.1 cells have the ability to differentiate into various types of mesenchymal cells. However, in culture, we observed only three distinct cell morphologies, multinucleate (giant), spindle or round cells. The round cells gave rise to round cell sarcoma and the spindle cells formed spindle cell tumors. A few multinucleate giant cells, are seen in some transformed cell populations. These populations consistently formed multinucleate giant cell sarcomas which we characterized as rhabdomyosarcoma.[16]

The Hadju[65] model of mesenchymal differentiation suggests that an undifferentiated mesenchymal cell such as MSU-1.1 can form various phenotypically distinct tumor types. If this were the case, one would expect to see marked diversity in tumor types such as leiomyocytic, angioblastic, hemangiocytic, lipoblastic, chondrocytic, hematopoietic, and osteoblastic tumors developing from this cell strain considering the large number of tumors examined. However, this clearly was not the case in our study. The modified Hadju model proposed by Brooks[64] offers a more plausible explanation for the results we observed. This model differs from the original in that it proposes an "intermediate

precursor" between the primitive cell and some differentiated phenotypes. This model also suggests a very close relationship between the intermediate precursor and the different phenotypes thus limiting to some degree the different phenotypic patterns the precursor intermediate cell is capable of achieving. Our data strongly support this hypothesis in that from examining 242 primary and 76 secondary tumors we found that a large percentage of the tumors were fibroblastic while only a small percentage were round or "rhabdomyosarcoma like". Based on morphology in this study, only three cell types could be clearly identified, the multinucleate cell (myoblast) the fibroblast and the undifferentiated "round cell". Tumors of osteocyte, chondrocyte, hematopoietic or endothelial origin were not seen. This observation supports the prediction made in the hypothetical model of mesenchymal differentiation proposed by Brooks[64] which states that most mesenchymal tumor phenotypes will be closely related when they are derived from the intermediate precursor cell. This is so, because the degree of differentiation of the intermediate precursor cell is very limited. We hypothesize that the MSU-1.1 cell strain has characteristics of the "primitive intermediate cell", with limited differentiating potential.

This study, reports for the first time on the observation of single and multiple phenotypes in tumors, induced by transformation of normal human fibroblasts by carcinogen treatment, or transfection of oncogenes. Since the oncogene transformed MSU-1.1 cells, formed tumors morphologically analogous to those tumors in humans, it is possible that the oncogenes that transformed MSU-1.1 cells, are activated in human tumors with a similar morphology.

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CONCLUSION AND FUTURE STUDIES

In summary, the results of the MT-*ras* study suggests that the level of H-*ras* oncoprotein expression plays an important role in the *in vitro* transformation of the normal human fibroblast cell strain MSU-1.1. H-*ras* transformed MSU-1.1 cells exhibit changes in cell morphology, anchorage independence and reduced growth factor requirements, while the normal parental MSU-1.1 cells do not have these characteristics. However, only high expressing H-*ras* oncoprotein transformed MSU-1.1 cells formed malignant tumors in nude mice. The growth of a malignant tumor e.g., fibrosarcoma, beyond 1-2 mm in diameter requires induction and maintenance of an angiogenic response (Folkman, J., 1990). Data reported in this dissertation supports this hypothesis in that, benign tumors were small and avascular while malignant tumors were large and well vascularized. Furthermore, the MT-*ras* study results raises the possibility that the level of H-*ras* oncoprotein expression may influence development of the malignant tumor phenotype. Because of the relationship between malignant transformation and angiogenesis a future project should explore this phenomenon by examining angiogenic factors such as vascular endothelial growth factor (VEGF) or vascular permeability factor (VPF). For example, using the MT-*ras* transformed cells experiments utilizing northern blot analysis or semi-quantitative RT/PCR could be done to determine if zinc induced *ras* mRNA expression causes a concomitant expression of VEGF/VPF mRNA expression. Since human umbilical vein endothelial cells (HUVEC) will proliferate in response to exogenous VEGF/VPF stimulation (Kohl, *et. al.* 1995) an indirect measure of *ras* induced VEGF/VPF activity could be also determined. For example, measuring the proliferative response of HUVEC cells grown in the presence of conditioned medium derived from zinc-stimulated MT-*ras* cells would be a potential end point. *In vitro* studies utilizing gene transfection methods and monoclonal neutralizing antibodies to VEGF/VPF could also be used to explore the contribution of VEGF/VPF to the progressive growth of solid

tumors through its promoting effects on tumor angiogenesis (Zhang, *et. al.* 1995; Kim, *et. al.* 1993). An important aspect of these future studies should determine if transfection and expression of VEGF/VPF gene in non-malignant MT-*ras* cells will cause the non-malignant cells to form malignant tumors when injected into nude mice.

It has been recently reported that the biologic activity of *ras* proteins is associated with enzymatic farnesylation a process which regulates transport of cytoplasmic *ras* protein to the cell membrane (Kohl, *et. al.*, 1993). It is conceivable that pharmacologic inhibition of farnesyltransferase should suppress the rapid growth of *ras* positive solid tumors by inhibiting the pro-angiogenic action mediated by the *ras* oncoprotein (James, *et. al.* 1993).

BPDE transformed cells that induced *ras* expressing malignant tumors must be examined to determine the specific alteration in the *ras* gene and these tumor cells should also be evaluated for expression of angiogenic factors such as basic fibroblast growth factor (bFGF) and VEGF/VPF. Analysis to define the *ras* genetic change is difficult as any number of codons as well as any one of the three *ras* genes could be altered.

I also classified the tumors induced by oncogene transfection and/or carcinogen treatment of MSU-1.1 cells. The results of this study indicate that different genes could be activated in the development of fibromas and sarcomas. In humans this finding is supported by results of clinical studies that examined oncogene expression in cases of fibromas and fibrosarcomas. A more detailed evaluation of the carcinogen transformed tumor cells is warranted because the present study is limited by the sensitivity of the techniques used for analysis of gene expression. The use of solution phase RT/PCR as well as *in situ* PCR techniques in such a study would provide the sensitivity and specificity needed to make comprehensive conclusions about the relationship between exposure to carcinogens and expression of specific genes in development of clinical tumors in humans. Further work also needs to be carried out to determine the correlation between specific oncogenes and certain tumor type e.g., rhabdomyosarcoma. Another tumor type that deserves close

examination is the malignant fibrous histiocytoma. To date activated oncogenes have not been described in this tumor type yet it is a relatively common soft tissue tumor in man.

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VITA

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He enrolled at Tuskegee Institute as an undergraduate student in 1978 and completed his bachelor's degree in 1981 and entered Tuskegee Institute School of Veterinary Medicine in 1982. After graduating in 1986 he worked as an intern in the Department of Pathology and Parasitology for one year before starting a residency in anatomic pathology at Michigan State University in 1987. At the completion of the two year residency he received an NIH postdoctoral fellowship to pursue a PhD in toxicologic pathology at Michigan State University. At the completion of the doctoral program the author joined SmithKline Beecham Pharmaceuticals as an experimental molecular pathologist with research focus in the areas of cardiovascular pathology and oncology.