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EVIDENCE THAT FORMATION OF NEUROFIBROSARCOMAS IN INDIVIDUALS WITH TYPE 1 NEUROFIBROMATOSIS IS ASSOCIATED WITH ACTIVATION OF GROWTH FACTOR PATHWAYS

By

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ABSTRACT

EVIDENCE THAT FORMATION OF NEUROFIBROSARCOMAS IN PATIENTS AFFECTED WITH TYPE 1 NEUROFIBROMATOSIS IS ASSOCIATED WITH ACTIVATION OF GROWTH FACTOR PATHWAYS

By

Jeremy W. Wray

One of the features of Type 1 Neurofibromatosis (NF1) is that affected individuals commonly form numerous benign neurofibromas, which arise from Schwann cells that surround peripheral nerves. Rarely, a neurofibroma cell undergoes malignant transformation giving rise to a neurofibrosarcoma. I used a reverse transcription-polymerase chain reaction (RT-PCR) assay to examine whether tumor cell lines derived from NF1-associated neurofibromas and neurofibrosarcomas express TGF-α and PDGF-B mRNA. The results of these experiments indicate the neurofibrosarcoma cells express elevated levels of TGF-α and PDGF-B mRNA relative to neurofibroma cells. Neurofibroma cells also express PDGF-B mRNA but at a lower level than that neurofibrosarcoma cells. Normal rat Schwann cells and human Schwann cells derived from neurofibromas do not express receptors for serum growth factors such as PDGF, therefore, I tested whether neurofibrosarcoma cells express such receptors by measuring proliferation of neurofibrosarcoma cells in response to PDGF, TGF-α and bFGF *in vitro*. These results indicate that some neurofibrosarcomas constitutively express one or more of the receptors for PDGF, EGF/TGF-α, and bFGF. This work also demonstrates that one of two neurofibrosarcoma

cell lines capable of growth in medium lacking exogenous growth factors is completely inhibited from growth in these conditions in the presence of 50 μg/ml of suramin. These results support a role for activation of growth factor pathways, including elevated expression of growth factors and growth factor receptors, in the abnormal proliferation of Schwann cells that occurs in NF1 patients.

This work is dedicated to my wife, Christine, and to our children.

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INTRODUCTION

Malignant cancer cells have acquired a number of characteristics including the ability to proliferate abnormally, invade tissue locally and/or form distant metastases (Vogelstein et al., 1988). These abnormal characteristics occur as a result of changes in the quantity or quality of expression of specific genes. The predominate theory to explain the acquisition of such changes is that they arise sequentially, and each change confers some growth advantage on the cell in which it occurs. Early observations recognized the association between certain behaviors or environmental exposures and specific types of cancer. However, it was not until the early 1980's that the genes which are altered in human cancers began to be identified. Much of the current research is aimed toward understanding the normal function of such genes and the mechanisms by which these genes become subverted in neoplasia.

Many of the genes associated with cancer that have been identified encode proteins that are involved in the transduction of mitogenic signals. Classes of proteins involved in signal transduction include: (1) growth factors, (2) growth factor receptors, (3) cytoplasmic proteins that transduce growth signals to cellular effector sites and, (4) nuclear factors that regulate the expression of genes involved in the mitogenic response. One of the primary goals of cancer research is to identify how the various mediators and regulators of signal transduction interact in normal cells and the mechanisms of abnormal signal transduction in cancer cells.

The growth of cells in multicellular organisms is highly regulated (reviewed by Cooper, 1992). In human adults, cells in some tissues divide continuously, whereas cells in other tissues divide rarely, if ever. For example, superficial cells of the gastrointestinal tract and the skin are continuously sloughed off, and constant, rapid cell division is required to replace these cells. Blood cells also divide continuously to replenish aging and dying cells. Conversely, neurons and cardiac muscle rarely if ever divide in mature adults. Understanding the mechanisms that control cell growth and division is central to understanding the uncontrolled growth characteristic of neoplastic cells.

Type 1 neurofibromatosis provides an opportunity to investigate neoplasia in cells that are predisposed to abnormal activation of the ras signal transduction pathway(s). Individuals affected with this disease have inherited one defective allele of the NF1 gene, which encodes a GTPase activating protein that is known as neurofibromin (DeClue et al., 1992). Neurofibromin, like other GTPase activating proteins, is involved in negative regulation of ras proteins. Therefore, individuals affected with NF1 are predisposed to neoplasia as a consequence of the inability to regulate ras normally.

NF1 patients typically develop many benign neurofibromas which can undergo malignant transformation to form a neurofibrosarcoma. Although the occurrence of neurofibrosarcomas is significantly elevated in NF1 patients compared to the general population, malignant transformation of a neurofibroma is nevertheless a rare event, given the number of neurofibromas from which they can arise. By studying cell lines that I have derived from neurofibromas and neurofibrosarcomas that occurred in individuals with NF1, I have been able to identify specific changes that are associated with malignant transformation of neurofibromas into neurofibrosarcomas.

The review of the literature in this dissertation has two aims. The first is to describe the evidence that has contributed to the current understanding of multistep carcinogenesis. The second aim will focus on signal transduction pathways that are mediated by ras proteins and the manner in which ras proteins are regulated in normal and abnormal cells. The second chapter is a paper that describes the evidence from my research indicating that specific growth factors, including PDGF-B and TGF-α, have a role in the pathogenesis of neurofibrosarcomas that occur in individuals with Type 1 neurofibromatosis, and is written in the format appropriate for *Carcinogenesis*. This paper (chapter 2) is the result of the majority of the research that I have done in my Ph.D. program. The last two sections are composed of minor chapters of additional research that I have done in The Carcinogenesis Laboratory. The third chapter describes the characterization of a cell line derived from a human malignant fibrous histiocytoma. The fourth chapter describes abnormal characteristics displayed by *normal* skin fibroblasts that were obtained from a 6 year old male with congenital myofibromatosis, a rare disease in which affected individuals are born with benign tumors. Finally, there is an appendix that describes the human tumor cell lines that I have established in culture in the course of my research.

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CHAPTER 1

LITERATURE REVIEW

I. Neoplasia is the Consequence of Alterations in DNA and/or DNA Expression

A. Tumors Have Many Changes

Each tumor that occurs in humans or other vertebrates is considered to be the consequence of qualitative and/or quantitative changes in the expression of specific genes. One predominate theory to explain the presence of multiple changes in tumor cells is that the changes arise sequentially, and each of change confers some growth advantage on the cell in which it occurs. Proliferation of a cell that has an advantageous change results in a large population of cells possessing the change. Such a population of cells can serve as a target for further rounds genetic change and subsequent selective growth. The accumulation of specific genetic changes in a single cell is considered to result in a neoplastic cell (Fearon and Vogelstein, 1990). Another theory explains the presence of multiple changes by assuming that infidelity of DNA replication is responsible for tumor oncogenesis and progression (Loeb et al., 1974). The following sections present evidence that has contributed to this current understanding of multistep carcinogenesis.

B. Early Observations

Evidence for a relationship between environmental and/or behavioral factors and the occurrence of cancer was recognized long before there was any understanding of the mechanisms of chemical carcinogenesis. For example, in 1775 Pott described a high frequency of scrotal cancer in males who had been employed as chimney sweeps when they were boys (Pott, 1775). Pott hypothesized that "The disease, in these people, seems to derive its origin from a lodgement of soot in the rugae of the scrotum,...". Nearly two centuries later benzo(*a*)pyrene was isolated from coal tar that is present in chimneys used to burn coal (Cook et al., 1933). Benzo(*a*)pyrene is a potent carcinogen and could have been the agent responsible for the high frequency of scrotal cancer that Pott observed in London chimney sweeps. Even prior to these observations by Pott, the Italian physician Ramazzini had recognized that nuns have a higher incidence of breast cancer than women in the general population (cf Boyland, 1980). Investigators now have some understanding of the protective effect that pregnancy-induced hormones exert on breast tissue. Another example of early insights into carcinogenesis came from Hill, who in his 1761 publication entitled, *Cautions Against the Immoderate Use of Snuff*, reported that users of tobacco snuff developed cancer of the nasal passage much more frequently than those who abstained (Redmond, 1970). The presence of carcinogens in tobacco and tobacco smoke, and their role in various types of cancer, is now well established. These investigators were some of the first to recognize that agents related to environment and/or behavior could elevate the risk for developing specific types of cancers.

C. Terminology of *In Vitro* transformation

The two general categories of changes that occur in multistep carcinogenesis have been classified as those that are *genetic* and those that are *epigenetic* (Barrett, 1987). By definition, genetic alterations describe "any change in phenotype which results from an alteration in primary DNA sequence. This change may be a single base pair change, a deletion, an insertion, a rearrangement or duplication of one or more base pairs, or loss or gain of an entire chromosome". An epigenetic change includes "any change in phenotype which does not result from an alteration in DNA sequence. This change may be stable and heritable, and includes alterations in DNA methylation, transcriptional activation, translational control, and post-translational modifications" (Barrett, 1987). However, these terms are often used imprecisely in the cancer literature. For example, abnormal elevation in transcription of a growth factor gene, defined as an *epigenetic* change according to Barrett, may in fact be the result of alteration of the DNA sequence in the promoter or enhancer region of the gene, and in that case it should be defined as a *genetic* change. To avoid confusion I will use the term *genetic* in reference to changes in gene sequence and changes in the level of gene expression, and I will describe the mechanism(s) of such changes when they are known.

D. DNA is the Target of Oncogenic Changes

Hypotheses that cancer originates from alteration of the genetic material were proposed prior to the discovery that genes are composed of DNA. The somatic mutation theory of carcinogenesis was first purposed by T.H. Boveri, considered by many to be the father of the somatic mutation theory of carcinogenesis (Boveri, 1929). His theory was based on the observation that cancer cells typically have abnormal, and often bizarre

chromosomes. Furthermore, he observed that the entire population of neoplastic cells in a given tumor usually shared the same chromosomal defects, which suggested that the defects were passed from a parental cell to its progeny. Based on cytogenetic analysis he proposed that heritable alterations in the chromosomes are responsible for cancer. In 1937 Furth and Kahn demonstrated that a single mouse leukemia cell injected into a syngeneic mouse caused the development of leukemia in rapid fashion, indicating that the malignant phenotype is stably heritable. These early studies suggested that cancer was the consequence of irreversible alteration of the genetic material.

The characterization of DNA as the genetic material suggested that the permanent effects caused by carcinogens occur at the DNA level. To test the hypothesis that DNA was the target of carcinogens, Brookes and Lawley (1964) investigated whether a positive correlation existed between the degree of binding of various carcinogenic hydrocarbons to DNA, RNA, and proteins and the degree of carcinogenicity of such compounds. They found no association between the carcinogenicity and binding of any of the hydrocarbons to protein or RNA. However, they found *significant positive correlation* between the binding of specific hydrocarbons to DNA and the carcinogenicity of the hydrocarbons (Brooks and Lawley, 1964).

In 1968 investigations by Maher et al. demonstrated a correlation between the degree of carcinogenic potency of various derivatives of 2-acetylaminofluorene (AAF) and *N*-methyl-4-aminobenzene (MAB) and the ability of each derivative to induce mutations in DNA. In these experiments DNA mutation was measured as a function of inactivation of transforming DNA isolated from SB 19, a strain of *Bacillus subtilis*. SB 19 DNA containing an intact tryptophan gene confers the ability to grow in the presence of indole to the recipient bacterial strain known as T3.

Treatment of strain SB 19 DNA with specific carcinogens reduced the ability of transforming DNA to confer growth proficiency to T3 recipient bacteria. Maher et al. found that when transforming DNA was treated with various derivatives of AAF and MAB, those compounds known to be the most carcinogenic *in vivo* were the most potent in: (1) reducing the transforming ability of transforming DNA, (2) elevating the frequency of mutations in transforming DNA, and (3) decreasing the buoyant density of transforming DNA as a result of covalent binding of the carcinogen residues to the DNA. Miller and Miller had earlier proposed that pro-carcinogens become ultimate carcinogens by metabolic conversion to electron-pair-deficient electrophilic reactants. In light of the studies of Maher et al. (1968), they extended that hypothesis to include the fact that such electrophilic metabolites bind readily to the genetic material to cause genetic damage.

One of the most convincing links between DNA damage and human carcinogenesis resulted from studies of cells obtained from patients affected with the autosomal recessive disease xeroderma pigmentosum (XP). These individuals are highly sensitive to sunlight exposure, and normal levels of exposure to sunlight results in early age, non-melanoma skin cancer (Johnson, 1982). Investigations of XP cells in culture demonstrated that XP skin fibroblasts are defective in repair of damage caused by UV irradiation (Cleaver, 1969). Treatment of XP cells with UV radiation resulted in a significant increase in mutation frequency compared to that of normal fibroblasts (Maher and McCormick, 1976). The conclusion from these studies is that the cells of individuals affected with XP have a decreased rate of UV-induced excision repair which in turn leads to an increased frequency of UV-induced mutations. This suggests that the genes responsible for skin cancer undergo an increased mutation

frequency, and this results in skin cancer in sunlight exposed areas of the body. In normal persons of light complexion who are exposed to sunlight for many years, skin cancer histologically similar to that found in XP patients occurs, but the cancer arises in these normal individuals much later in life than it does in XP patients.

II *In Vitro* Studies that Support Multistep Carcinogenesis

In vitro transformation experiments have been valuable in the identification of those genes altered in carcinogenesis as well as in understanding the biochemical role of such genes in normal and neoplastic cells. The following terminology used in *in vitro* transformation was agreed upon by the Tissue Culture Association (Schaeffer, W.I., 1990) and will be used in this dissertation. "*In vitro* transformation" refers to any heritable phenotypic change that can be observed and/or measured. This differs from "*in vitro* neoplastic transformation" which refers to *in vitro* acquisition of the ability to form benign or malignant tumors in the appropriate experimental animal. The term "*in vitro* malignant transformation" is reserved to describe cells that have acquired, in culture, the ability to form a tumor with malignant characteristics, including the ability to invade host tissue locally and/or metastasize within the host animal.

A. Investigations of Rodent Cells *In Vitro*

1. Spontaneous transformation

Gey was the first to report that spontaneous malignant transformation of rodent cells could occur *in vitro*. He observed that rat fibroblasts propagated in cell culture for a period of time formed

sarcomas when they were injected into rats (Gey, 1941). Shortly thereafter, Earle reported that mouse fibroblasts undergo spontaneous malignant transformation in culture, albeit at a lower frequency than those treated with carcinogen (Earle, 1943). Later studies demonstrated that the *in vitro* environments used by Earle and Gey were inherently genotoxic as a result of factors such as high oxygen concentrations and the presence of visible light (Parshad et al., 1977). Improvements in cell culture techniques have reduced the frequency of spontaneous transformation that occurs in rodent cell culture systems (Sanford and Evans, 1982).

2. Carcinogen-Induced Transformation

Carcinogen-induced *in vitro* transformation of Syrian hamster embryo (SHE) cells was first described by Berwald and Sachs (1963, 1965). When SHE cells plated at cloning density were treated with either 3-methylcholanthrene or benzo(*a*)pyrene, some of the clones were found to consist of morphologically altered cells. When populations of carcinogen-treated SHE cells were continuously propagated in cell culture some of the cells escaped senescence. Untreated SHE cells did not escape senescence in these experiments.

Barrett and Ts'o demonstrated that when SHE cells treated with benzo(*a*)pyrene were routinely subcultured, they expressed properties of transformed cells, including morphological transformation, fibrinolytic activity, anchorage independence (growth in soft agar), and the ability to form tumors in athymic mice (1978). Furthermore, these changes occurred in a sequential manner. Morphological transformation occurred within one week of carcinogen treatment. Enhanced fibrinolytic activity followed two to three weeks after carcinogen treatment. Six to 15 weeks after (32 to

75 population doublings) after carcinogen treatment, cells acquired the ability to form large colonies in soft agar.

The ability of the transformed SHE cells to grow in soft agar correlated with malignant transformation. Eight different populations of carcinogen-treated SHE cells that had become morphologically transformed, but had not yet become anchorage independent, did not produce tumors in neonatal hamsters. However, carcinogen-treated SHE lines that had attained anchorage independence formed tumors in 100% of the neonatal hamsters into which they were injected. SHE cells that had spontaneously immortalized in cell culture (occurs at a low frequency in control cells), but were not anchorage independent, did not form tumors in athymic mice. However, one spontaneously immortalized SHE cell line became anchorage independent at passage 37, and when injected into neonatal hamsters, this cell line produced tumors in three of six animals (Barrett and Ts'o, 1978). The observation that SHE undergo several discernable phenotypic changes on the path towards malignancy suggests that multiple genetic changes are required to achieve the malignant state.

3. Oncogene-Induced Transformation

Investigations of the RNA virus known as the Rous sarcoma virus (RSV), gave the first direct evidence that alteration of a normal cellular gene could contribute to neoplastic transformation. RSV is a transforming retrovirus and can neoplastically transform the cells that it infects. Bishop and his colleagues showed that homologs of the transforming sequence of RSV were present in the genomes of normal avian (Stehelin et al., 1976) and mammalian (Spector et al., 1978) cells. The finding that diverse species carry normal genes homologous to the viral *src* transforming gene of RSV suggested that normal genes have the

potential to participate in carcinogenesis if they are altered, and that RSV obtained the *src* gene by transduction of the host *src* sequence after infection of a host cell (Bishop, 1983). Other investigations revealed that many retroviral oncogenes contain sequences homologous to normal genes present in several animal species including humans (reviewed by Bishop, 1983). The normal cellular genes that correspond to the transforming sequences of retroviruses were called protooncogenes to distinguish them from the oncogenes of retroviruses.

The identification of protooncogenes in human cells led investigators to ask whether such genes played a causal role in human cancers. One strategy to detect whether activated protooncogenes were present in human tumors was to introduce DNA isolated from a human tumor into non-cancer cells and assess whether the tumor DNA was able to transform the recipient cells. Such an assay had been used to identify the oncogenic sequences of transforming retroviruses. The most common assays utilized the immortalized murine cell line NIH 3T3 (Andersson et al., 1979; Copeland and Cooper, 1979, Lowy et al., 1978). Weinberg and his colleagues demonstrated that transfection of DNAs isolated from human, murine, rabbit, and rat tumor cell lines induced transformation of NIH 3T3 cells (Shih et al., 1981). Transfection of DNAs from two human bladder carcinoma cell lines also induced transformation of NIH 3T3 cells (Krontiris and Cooper, 1981). Analysis of the NIH 3T3 cells transformed by DNA prepared from these human tumors revealed that the sequences responsible for transformation of the NIH 3T3 cells were homologous to the *ras* genes of the Harvey and Kirsten sarcoma viruses (Der et al., 1982; Parada et al., 1982 Dahr et al., 1982). Subsequently, transforming DNA sequences from numerous human cancers were characterized and matched to their transforming retroviral homologs (Land et al., 1983; Slamon et al.,

1984; Bishop, 1987). These studies demonstrated that activated protooncogene sequences were present in human malignancies and strongly suggested that alterations in specific protooncogene sequences contributed to the genesis of neoplastic cells in humans.

B. Investigations of Human Cells *In Vitro*

Unlike rodent cells, normal diploid human fibroblasts have never been observed to undergo spontaneous transformation *in vitro* . When normal human foreskin fibroblasts are cultivated *in vitro* they undergo approximately 50 population doublings (Hayflick and Morehead, 1961) after which they cease cell division. Normal diploid epithelial cells divide only a few times in cell culture and also have not been observed to undergo spontaneous transformation *in vitro* (Chang, 1986). Although rodent cell models have been an indispensable tool in advancing the understanding of carcinogenesis, the differences between rodent cells and human cells suggested the need for human cell systems to investigate carcinogenesis *in vitro*.

1. Carcinogen Experiments using Normal Human Cells

In contrast to the numerous studies that describe *in vitro* malignant transformation of normal rodent cells, there are only a few reports of carcinogen-induced or spontaneous *in vitro* malignant transformation of normal human cells, and these have not been verified (see review by McCormick and Maher, 1988 and McCormick et al., 1988). The most cited example is that of Kakunaga (1978), who reported that treatment of the normal human fibroblast cell line KD with a single dose of carcinogen, resulted in malignantly transformed cells. Because similar experiments failed to reproduce the results of Kakunaga, attempts were made to

determine if the malignant cells arose from the normal parental cell line. Investigations of enzyme patterns, HLA antigens, restriction fragment length polymorphisms, and marker chromosomes clearly showed that the malignant cells could not have arisen from cell line KD. Further analysis revealed that the isoenzyme pattern of the malignant cells was identical to the human fibrosarcoma cell line 8387 (McCormick et al., 1988). These results strongly suggest that the cell line 8387 cross-contaminated the culture of carcinogen treated KD cells.

In 1978 Milo and Dipaolo selected carcinogen-treated normal human fibroblasts for their ability to form colonies in soft agarose (Milo and Dipaolo, 1978), and reported that such cells formed progressively growing undifferentiated mesenchymal tumors when injected into athymic mice. However, similar attempts by other laboratories to generate malignant cells from normal human cells by carcinogen treatment *in vitro* have not been successful (McCormick and Maher, 1989).

Investigators have achieved *in vitro* morphological transformation, but not malignant transformation, of normal, finite life-span human cells by carcinogen treatment *in vitro*. In each of these studies, normal cells were exposed to a carcinogen, then cultured under conditions in which transformed cells could be distinguished from non-transformed cells. For example, when normal human cells were treated with MNNG are subsequently grown in low serum conditions, they gave rise to areas of focus formation (focal piling up of cells) (DeMars and Jackson, 1977). Though the focus-derived cells were morphologically transformed, they did not form progressively growing tumors when they were injected into athymic mice. Similarly, normal human foreskin fibroblast treated with propane sultone attained the ability to form colonies in soft agar, but failed to form tumors in athymic mice (Silinskas et al., 1981). Other investigators

generated anchorage independent cells *in vitro* by treatment with ^{60}Co radiation (Wang et al., 1986) or heavy metals (Biedermann and Landolph, 1988), but anchorage independent cells in these investigations also produced no tumors in athymic mice. One recent study demonstrated that human mammary cells became malignantly transformed after repeated exposures to ionizing radiation followed by suitable selection after each treatment (Wazer et al.,1994). This is the first report of *in vitro* generation of malignant cells from normal human cells by carcinogen treatment alone.

2. Transfection of Normal Human Cells with Oncogenes

Experiments designed to transform normal, finite life-span cells into neoplastic cells by oncogene transfection have also generally been unsuccessful. Transfection of *ras* oncogenes did not transform normal finite life-span human fibroblasts to the malignant state even though Southern analysis demonstrated the presence of the activated *ras* oncogene in the genome of the transfectants (Sager et al., 1983). When an oncogenically activated, over-expressed H-*ras* gene was transfected into normal human fibroblasts, the cells formed foci of morphologically transformed cells, but such cells did not form malignant tumors in athymic mice (Hurlin et al., 1987). Investigations in which other oncogenes including v-*sis*, c-*sis*, H-*ras* (Fry et al., 1986, 1988), or N-*ras* (Wilson et al., 1989) were transfected into normal human diploid fibroblasts also generated no malignant cell strains.

3. Infinite Life Span Human Cell Strains

One possible explanation for the inability of *ras* oncogenes to malignantly transform normal human cells is that the cells did not have

the ability to continuously replicate even though they were morphologically transformed. This hypothesis suggested that what was needed to obtain malignantly transformed human cells *in vitro* by transfection of *ras* oncogenes was an infinite life-span human cell line that could continue replicating indefinitely. Cell lines with an infinite life-span could theoretically survive long enough to acquire all of the changes necessary to become malignant. Namba and his colleagues were able to generate infinite life-span fibroblast lines by repeated ^{60}Co gamma exposure or repeated chemical carcinogen treatment of normal human fibroblasts (Namba et al., 1981, 1985, 1988). The resulting cell lines were not malignant, but did exhibit many features tumor-derived cells, including altered cell morphology and reduced requirements for serum or growth factors in cell culture. Similar experiments generated immortal cell lines from normal human mammary epithelial cells (Stampfer and Bartley, 1985). Such experiments have not been repeated by other investigators. However, there is no doubt of their validity since molecular techniques have demonstrated that the immortalized cells arose from the parental cells (McCormick and Maher, 1988)

The infinite life-span human cell strains just described have already acquired many features of transformation, such as anchorage independence and the ability to grow in the absence of exogenous growth factors. Because of their partial transformation, they are of limited utility in investigating malignant transformation. McCormick and his colleagues took another approach in order to create an infinite life-span, human cell strain which was otherwise normal and therefore more useful for studying *in vitro* malignant transformation. They transfected normal human foreskin fibroblasts with the v-*myc* oncogene (Morgan et al., 1991). This strategy was used because of other investigations which demonstrated that

rat embryo fibroblasts undergo spontaneous immortalization at a very low frequency, but when such fibroblasts were transfected with *myc* oncogenes the frequency of immortalization increased significantly (Land et al., 1986). The v-*myc* construct employed by Morgen et al. (1991) also contained a gene coding for resistance to geneticin, so transfectants from the human fibroblasts could be selected for drug resistance. Five independent clonal populations of geneticin resistant transfectants were selected and were serially passaged to the end of their life-span. All five clonal populations of v-*myc*, geneticin resistant cells eventually sensesced. However, in one of the senescing populations a few cells (perhaps a single clone) continued to proliferate. This cell strain has continued to divide beyond 200 population doublings and is known as MSU-1.1. The finding that the vast majority of that clonally-derived population senesced indicates that an additional change or changes are required, in conjunction with v-*myc* expression, to cause a normal human fibroblast to become immortal. Isozyme and restriction fragment length polymorphism patterns of the immortal MSU-1.1 strain are identical to that of the normal parental fibroblast cell line from which MSU-1.1 was generated demonstrating that MSU-1.1 arose from a normal human fibroblast (Morgan et al., 1991).

4. Neoplastic Transformation of Infinite Life-Span Human Cell Strains

There are a number of studies in which immortalized human cell lines have been neoplastically or malignantly transformed by oncogene transfection. O'Brien et al. (1986) found that when SV40 immortalized fibroblasts were infected with the Kirsten murine sarcoma virus, which carries the K-*ras* oncogene, they acquired the ability to form small, static nodules when injected into athymic mice. Similar experiments by

Rhim and his colleagues (1985) generated cells that formed malignant tumors in athymic mice. Namba et al. (1988) found that a carcinogen immortalized human cell line became malignantly transformed when it was transfected with the K-*ras* oncogene. (Namba et al., 1988). These investigations could be interpreted to mean that some immortalized cells need only a single change to become neoplastic. However, SV40 immortalized cell lines typically express features of transformation in addition to infinite life-span, such as anchorage independence and growth factor independence, and the immortalized fibroblasts generated by Namba occurred only after several exposures to carcinogen treatments. The implication is that each of these cell strains has already acquired many of the changes necessary to become a neoplastic cell in addition to the change(s) leading to immortality. Furthermore, expression of viral oncogenes by transfected cells can be interpreted to consist of more than a single change. This point is illustrated by studies comparing constructs that express either the H-*ras* (Hurlin et al., 1989) or the N-*ras* (Wilson et al., 1990) oncogene at either high or low levels. When the immortal human fibroblast line MSU-1.1 was transfected with low expression mutant *ras* oncogene constructs, the cells were not transformed to malignancy, whereas transfection with mutated *ras* engineered for high expression caused MSU-1.1 cells become malignantly transformed. These observations suggest that at least two changes, high expression and mutation, are necessary to cause a c-*ras* protooncogene to acquire the transforming capability of a v-*ras* oncogene.

There are a few examples of infinite life-span human cell lines that have been malignantly transformed by carcinogen treatment. SV40-immortalized human fibroblasts treated with various carcinogens resulted in strains that were malignantly transformed (Rhim et al., 1985, 1990;

Reznikoff et al., 1988). When the infinite human fibroblast line, MSU-1.1, was treated with a single treatment of (+/-)-7ß,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), the ultimate metabolite of benzo[*a*]pyrene, then replated in low serum conditions and allowed to grow to confluence, foci of morphologically transformed cells appeared (Yang et al., 1992). A number of cell strains from these independent foci, were assayed for various features of transformation including the ability to form malignant tumors in athymic mice. Three of the eight focus-derived strains formed progressively-invasive malignant tumors in athymic mice, one strain formed a benign tumor, and others did not produce tumors at all. Yang et al. demonstrated that focus formation alone does not predict neoplastic transformation. Only focus-derived cells that were also anchorage independent and growth factor independent were neoplastically transformed. These results demonstrate that two or more changes are necessary for malignant transformation of fibroblast strain MSU-1.1.

III. *In Vivo* Models of Multistep Carcinogenesis

A. Mouse Skin Carcinogenesis

1. The Two-Step Model

In 1941 Berenblum reported that co-treatment of mouse skin with croton oil and the carcinogenic hydrocarbon, benzo(*a*)pyrene, resulted in a significant increase in the frequency of benign and malignant neoplasms compared to that found following benzo(*a*) pyrene treatment alone. Berenblum tested croton oil and other non-carcinogenic agents as co-carcinogens because of the studies of Rous and his associates which suggested a causative role for "irritation", by chemical agents or

physical injury in carcinogenesis (Rous and Kidd, 1941; Friedwald, and Rous, 1944). In 1944 Mottram showed that a single, sub-carcinogenic application of 7,12-dimethylbenz(a)anthracene (DMBA), another carcinogenic hydrocarbon, followed by repeated treatments with croton oil, also resulted in production of multiple skin tumors in mice. These and other experiments led to the two-stage hypothesis of carcinogenesis: initiation followed by promotion (Boutwell, 1974; Slaga, 1983). In mouse epidermal carcinogenesis, initiation consists of the application of a sub-carcinogenic dose of carcinogen to the skin. Promotion refers to multiple treatments of the skin with a tumor promoter after initiation has occurred. Promoters are not carcinogens and do not cause tumors even when applied at high doses over a long period of time. The promotor in croton oil was found to be the phorbol ester, 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA) (Hecker,1968).

In the mouse skin model, initiation followed by successive rounds of promotion results in the development of multiple benign papillomas, a few of which develop into carcinomas after 20-60 weeks of TPA treatment (Slaga, 1983). These observations imply that the initial carcinogen treatment results in a number of initiated cells, each of which has an irreversible genetic change. Promoter-induced proliferation results in clonal expansion of these initiated cells, resulting in papillomas. If promotor treatment is stopped, the papillomas usually regress. However, if promotor treatment is continued some of the papillomas develop into carcinomas. As a result of this observation the term *progression* has been added to *initiation* and *promotion* to describe the events following promotion which cause benign tumors (papillomas) to give rise to malignant tumors (Burns et al., 1978; Albert et al., 1979). Therefore, the mouse

skin model suggests that there are at least three steps in the genesis of a malignant cell from a normal cell.

Subsequent investigations using the mouse model further support the hypothesis that mouse epidermal carcinogenesis consists of multiple steps. Shubik (1950) described four distinct phenotypic stages in progression toward malignancy. These stages included (1) small tumors that appeared and were present for at least four weeks but then regressed and did not reappear, (2) papillomas that appeared and remained at a fixed size during the extent of the experiment, (3) papillomas that grew progressively during the extent of the experiment but did not attain malignancy and (4) those that progressed to malignancy. Hennings et al. (1983) used a variation in the standard mouse skin carcinogenesis protocol. In this work initiation and promotion steps were followed by an additional carcinogen treatment, resulting in a significant increase incidence of carcinomas. This implies that carcinomas arise from benign papillomas as the result of an additional DNA damaging event(s). In the classical model, the additional genetic event(s) is spontaneous and occurs during TPA induced proliferation. The rarity of a spontaneous mutation is illustrated by the low frequency of papillomas that progress to carcinomas. The second carcinogen treatment in the modified protocol magnifies the number of carcinomas that occur by inducing DNA damaging events in papilloma cells that already contain the first genetic change(s). This work to refine the mouse skin model supports the hypothesis that multiple steps are required for the genesis of a malignant cell.

2. Oncogenes in Mouse Skin Carcinogenesis

The discovery of the role of retroviral oncogenes in animal carcinogenesis and the finding that protooncogenes are often activated in human and animal malignancies led investigators to hypothesize that known oncogenes might become activated during mouse epidermal carcinogenesis. When mouse epidermal cells were stably infected with a retrovirus that contained the H-*ras* oncogene, they did not express any phenotypic change in the absence of promoter treatment. But when the infected cells were treated with TPA, papillomas occurred, some of which progressed to malignancy (Brown et al., 1986). Therefore, *H-ras* seems capable of initiating mouse skin cells but other changes are necessary for the initiated cells to become neoplastic. Examination of the viral integration sites of these epidermal papillomas demonstrated that the papillomas were polyclonal whereas the carcinomas were monoclonal in origin. Since each monoclonal carcinoma arises within a papilloma population, the multistep model of carcinogenesis is validated. In this model, a single cell undergoes a genetic change which confers that cell with a selective advantage, leading to clonal expansion of that cell. Such a clone of cells then serves as a target for further genetic change and selection.

Evidence that introduction of *ras* genes could initiate mouse skin cells led to investigations to determine whether *ras* or other known cancer genes were activated in papillomas and/or carcinomas arising in carcinogen-induced mouse skin tumors. Quintanilla et al. (1986) found that papillomas induced by DMBA and promoted with TPA nearly always contain an A --> T transition at the second position of codon 61 of the H-*ras* gene. Bianchi et al. (1990) and Brember and Balmain (1990)

demonstrated that amplification and/or over-expression of an activated H-*ras* gene, possibly in association with loss of the remaining normal H-*ras* allele, was associated with progression of papillomas to carcinomas. It therefore appears that *ras* activation can be responsible for two different aspects of carcinogenesis in mouse skin.

Recent studies demonstrate that tumor suppressor genes also have a role in mouse skin carcinogenesis. Alterations in the structure or expression of the *p53* tumor suppressor gene in benign or malignant cells derived from carcinogen-induced mouse skin tumors suggest that it is involved at a later stage in mouse skin carcinogenesis. *p53* expression was normal in papilloma-derived cells and well differentiated carcinoma cells, whereas a high frequency of undifferentiated carcinomas were found to contain *p53* gene alterations including loss of heterozygosity at the *p53* locus or point mutations in one or both *p53* alleles (Ruggeri et al., 1991). Many of the undifferentiated carcinomas lacked the p53 protein, which is in agreement with the gene data.

B. Human Colorectal Carcinogenesis

The mouse skin carcinogenesis model provided the first well-characterized example of *in vivo* multistep carcinogenesis. Similar studies in humans could not be carried out because of the obvious ethical considerations. Nevertheless, the accessibility of normal and neoplastic colorectal tissue via colonoscopy has provided investigators with the opportunity to study genetic changes that have occurred in tissues obtained from each of the identified stages in human colorectal tumorigenesis. Analysis of the histological stages that occur in human colorectal carcinogenesis have identified a progression of distinct histological phenotypes including: (1) hyperplastic growth of apparently

normal colonic epithelial cells, (2) dysplastic hypertrophic lesions, (3) small benign adenomas that increase in size and abnormal cellular features, (4) malignant foci embedded within adenomas, (5) fully invasive malignant lesions, and (6) carcinomas that have invasive and metastatic capability (Haggitt and Reid, 1986). It is thought that most colorectal carcinomas, sporadic or otherwise, arise from hyperplastic colonic epithelial cells in a sequence similar to that just described (Sugarbaker et al., 1985).

Several inherited syndromes have been identified that predispose affected individuals to colorectal carcinoma including familial adenomatous polyposis coli, hereditary nonpolyposis colorectal cancer syndrome, Peutz-Jeghers syndrome, juvenile polyposis syndrome, Gardner's syndrome, Muir-Torre syndrome and Cowden's disease (Haggitt and Reid, 1986; Hamilton, 1993). Furthermore, inflammatory bowel diseases, including Crohns disease and ulcerative colitis, elevate the risk of colorectal carcinoma in affected individuals, presumably through exposure of the colonic epithelial cells to the effects of chronic inflammation (Glickman, 1987). Investigation of neoplastic tissues obtained from individuals affected with familial adenomatous polyposis coli (FAP), hereditary nonpolyposis colorectal cancer (HNPCC) syndromes, as well as sporadic colorectal cancers, have been valuable in identifying and understanding genetic changes that occur in human colorectal carcinogenesis.

1. Familial Adenomatous Polyposis Coli

Individuals affected with the syndrome known as familial adenomatous polyposis coli (FAP) develop multiple benign colorectal adenomas early in life, some of which inevitably progress to colorectal carcinomas if the

colon is not removed (Okamoto et al., 1988). This syndrome is transmitted in an autosomal dominant fashion to offspring, and is relatively rare, accounting for only about one percent of colorectal malignancies. However, since each of the various stages observed in colorectal carcinogenesis can often be identified in the colon of an affected individual, and because clinical specimens are readily available, FAP provides a unique opportunity to study *in vivo* human multistep carcinogenesis.

The pattern of inheritance and disease progression of FAP suggests that affected individuals have inherited a constitutional genetic defect that predisposes their colonic epithelial cells to the initial phenotypic changes (such as hyperplasia) on the path toward colon carcinoma (Fearon et al., 1987). Leukocytes obtained from individuals with FAP were immortalized by Epstein Barr virus, then linkage analysis was performed, localizing the germ-line defect to the long arm of chromosome five (Leppert et al, 1987). Investigations of germ-line mutations in numerous patients in several different affected families led to mapping and sequencing of the gene, which is known as the *APC* gene (Nishisho et al., 1991, Groden et al., 1991).

Prior to the cloning of the *APC* gene, Vogelstein (1988) had hypothesized that the loss of a single *APC* allele, either as a germ-line or somatic mutation, results in widespread epithelial hyperplasia, and that such hyperplasia is the foundation from which hundreds to thousands of benign adenomas arise. These predictions were based on studies of allelic deletions in adenomas and carcinomas of the colon in normal individuals as well as those affected with FAP. Once the *APC* sequence was determined, sequencing experiments of the entire *APC* coding region in immortalized leukocytes from individuals affected with FAP demonstrated

that 59 of 79 unrelated kindreds carried germ line *APC* gene mutations (Miyoshi et al., 1992), confirming it as the defective gene in the majority of individuals with a clinical diagnosis of FAP. Sequencing experiments of colorectal neoplasms in normal individuals demonstrated that 63% of adenomas and 60% of the carcinomas that occurred sporadically also contained mutations in the *APC* gene (Powell et al., 1992). Therefore, *APC* gene defects are associated with carcinogenesis in a majority of sporadic colorectal tumors as well as those that occur in FAP affected individuals. Furthermore, since the frequencies of *APC* mutations in colorectal adenomas and carcinomas are essentially equal, these findings also suggest that *APC* gene defects are early events in colorectal carcinogenesis.

Analysis of the predicted protein structure of the *APC* gene product at the time that the sequence became known showed no specific homology with any known protein (Nishisho et al., 1991, Groden et al., 1991). However, sequencing of the APC gene in individuals affected with FAP revealed that greater than 90% of such patients have mutations that result in a messenger RNA that prematurely terminates during translation , suggesting that the carboxy terminus of the APC gene product is required for normal function (Miyoshi et al., 1992). Antibodies targeted against various regions of the predicted amino acid sequence of the APC protein demonstrated that normal cells in individuals affected with FAP contain both normal and truncated APC proteins. Cell lines derived from sporadic and FAP colorectal neoplasms contained no normal APC protein, and 75% of such cell lines contained truncated APC protein (Smith et al., 1993). Normal APC protein was detected in 40/40 human tumor cell lines derived from neoplasms that occurred in organs other than the colon in normal individuals. These studies demonstrate that the loss of the normal APC

gene product is associated with the formation of most, if not all, colorectal malignancies, but do not appear to be involved in sporadic malignancies arising in other organs.

Recent studies have given some insight into understanding the normal and abnormal function of the APC protein. Vogelstein and his colleagues showed that fusion proteins containing the first 171 amino acids of the APC protein bind to the normal APC protein *in vitro* (Su et al., 1993a). Experiments designed to detect proteins that immunoprecipitate with the APC protein revealed that α- and ß- catenin coimmunoprecipitate with the APC protein (Rubinfeld et al., 1993; Su et al., 1993b). The catenins are proteins that interact with the cell adhesion molecule known as E-cadherin. The results suggest two important points: (1) that defective APC protein may be able to bind and to some degree interfere with normal APC protein function (dominant-negative effect) and (2) that the normal APC protein is involved in cell adhesion.

2. Hereditary Non-Polyposis Colon Cancer

Hereditary non-polyposis colorectal carcinoma (HNPCC) is an autosomal dominantly inherited predisposition to colorectal cancer that was first described by Warthin in 1913. This disease is clinically diagnosed in individuals who develop a colorectal carcinoma and fulfill each of the following clinical criteria including: (1) three or more relatives with histologically verified colorectal carcinoma, one of whom is a first degree relative of the other two; (2) colorectal carcinoma involving at least two generations; and (3) one or more colorectal carcinoma cases diagnosed before age 50 (Vasen et al., 1991). Other characteristics of this disease include a tendency to develop carcinomas in the proximal (or right) colon, occurrence of malignancies at an early

age, and predisposition to malignancy in other organs including the ureter, kidney, stomach, small bowel, ovary, pancreas, biliary tract, skin, larynx and blood (Lynch et al., 1993). This syndrome is much more common than FAP and is thought to account for five to 15% of all colorectal carcinomas (Hamilton, 1993).

Data from linkage analyses demonstrated that the HNPCC phenotype is closely linked to a marker on chromosome two in some families affected with HNPCC (Peltomäki, et al., 1993) and to a marker on chromosome three in other families affected with HNPCC (Lindblom et al., 1993). Comparison of the types of genetic alterations in colorectal neoplasms that occurred in individuals affected with HNPCC and in those that occurred in non-affected individuals revealed a startling difference in the nature of the mutations. Analysis of oncogenes or tumor suppressor genes known to be frequently altered in colorectal cancers, including K-*ras*, P53, and *APC*, demonstrated that each of these genes were mutated slightly more frequently in HNPCC colorectal neoplasms than in non-HNPCC neoplasms. What was most interesting and unexpected was that errors in DNA replication appeared to account for an unusually high percentage of the mutations that occurred during colorectal carcinogenesis in HNPCC individuals (Aaltonen et al., 1993). Based on the known sequences of mismatch repair genes in other species, investigators found the human HNPCC gene on chromosome two to be homologous to the yeast *hMSH2* gene (Fishel et al., 1993; Leach et al., 1993). A short time thereafter, similar strategies were applied in the identification of the *hMLH1* gene on human chromosome three (Bronner et al., 1994; Papadopoulos et al., 1994). The implications of these findings is that germ-line defects in either of these genes cause defective mismatch repair during DNA synthesis.

Mutations in oncogenes or tumor suppressor genes that occur as the result of this defective mismatch repair lead to colorectal neoplasia.

3. Other genetic Changes Associated with Colorectal Neoplasia

Mutations in *ras* genes have also been implicated in colorectal tumorigenesis . Approximately 50% of benign adenomas larger than one centimeter and 50% of colorectal malignancies contain *ras* gene mutations, whereas fewer than 10% of adenomas smaller than one centimeter have *ras* mutations (Bos et al., 1987, Forrester et al., 1987, Farr et al., 1988). Therefore, it does not appear that *ras* mutations are often the initial change in colorectal carcinogenesis. Nevertheless, since the frequency of *ras* mutations does not increase with the occurrence of malignant tumors, it appears that *ras* mutations are usually a relatively early change in colorectal carcinogenesis. Further evidence that *ras* mutations play a role in the early stages of colorectal carcinogenesis comes from the study of a cell line established from a colorectal adenoma that occurred in a person affected with FAP. This cell line, designated PC/AA, has a mutation in K-*ras* codon 12 (Farr et al., 1988). Both early and late passage PC/AA cells were not tumorigenic when injected into athymic nude mice, even though colorectal carcinoma cell lines typically formed progressively growing malignant tumors in athymic mice.

Investigations of p53 gene mutations suggest that loss of p53 function is a late event in colorectal tumorigenesis. Vogelstein and his colleagues used restriction fragment length polymorphism analysis to detect loss of heterozygosity at the p53 locus on chromosome 17p (Fearon et al., 1987). They obtained both normal and neoplastic tissues from a number of patients. Tumor samples included in the study were those from individuals whose normal cells were heterozygous at the *p53* locus. Their

results showed that 76% (25/33) of informative colorectal carcinomas had allelic deletions of the *p53* gene, whereas only 3% (1/30) of the precursor colorectal adenomas showed loss of heterozygosity at the *p53* locus.. These findings suggest that loss of p53 function is often involved in progression of colorectal adenomas to carcinomas.

Other genetic changes frequently associated with human colorectal carcinogenesis include allelic deletion of chromosome 18q (Vogelstein et al., 1988), elevated expression of the c-*myc* gene (Erisman et al., 1988) and DNA hypomethylation (Goelz et al., 1985). A number of other, less frequent changes have also been described including the allelic deletions of other chromosomal sites (Fearon & Vogelstein, 1990). Although there appears to be a preferred order in the sequence in which the genetic events occur in colorectal carcinogenesis, it is the accumulation of changes that results in a neoplastic cell. Furthermore, it does not appear that any of the specific genetic defects identified so far are an absolute prerequisite in colorectal carcinogenesis. For example, although p53 gene defects have been identified in a majority of colorectal carcinomas, a portion of such tumors have normal p53 alleles (Fearon et al., 1987). Presumably there are other genes that participate in colorectal carcinogenesis.

IV. Ras proteins are key mediators of mitogenic signals in normal and neoplastic cells

A common characteristic of the genes implicated in cancer is that most, if not all, encode proteins that promote or regulate cell growth and division. Growth of cells in multicellular organisms is highly regulated.

In human adults, cells in some tissues divide continuously, whereas cells in other tissues divide rarely, if ever. For example, superficial cells of the gastrointestinal tract and the skin are continuously sloughed off, and constant, rapid cell division is required to replace the cells that are lost. Blood forming cells also divide continuously to replenish aging and dying cells. Conversely, neurons and cardiac muscle cells rarely if ever divide in mature adults (reviewed by Cooper, 1992). Understanding the mechanisms that control cell growth and division is central to understanding the uncontrolled growth characteristic of neoplastic cells. Many of the proteins involved in the mitogenic signal transduction have been identified, but the precise mechanisms of signal transduction are not completely understood. It is hoped that the discovery of the details of mitogenic signalling mechanisms in normal and neoplastic cells will reveal differences that can be used as targets for novel cancer treatments.

Classes of proteins involved in signal transduction include: (1) growth factors, (2) growth factor receptors, (3) cytoplasmic proteins that transduce growth signals to cellular effector sites and, (4) nuclear factors that regulate the expression of genes involved in mitogenic responses. The replication of normal cells is initiated by mitogenic growth factors (Deuel et al., 1988). Growth factors bind to specific cytoplasmic or transmembrane receptors that become activated, and as a result can transduce the mitogenic signal to effector proteins. The signal eventually reaches the nucleus where specific genes are either turned on or off in such a fashion that the cell becomes committed to DNA synthesis and cell division. The multistep hypothesis of carcinogenesis considers that a neoplastic cell is the result of a series of genetic alterations. It is therefore plausible that synergistic or additive defects in proteins involved in one or more signal transduction pathway,

including genes encoding proteins that inhibit growth and those that promote growth, contribute to cells becoming neoplastic. The following sections describe some of the details of signal transduction, with an emphasis on the regulation and function of ras proteins and their pivotal role in growth factor-initiated signalling pathways.

Viral *ras* oncogenes were first identified as the transforming sequences of retroviruses. Shortly thereafter, cellular *ras* genes were identified, and investigation of human tumor DNA identified transforming sequences that were altered forms of c-*ras* genes (for review see Bishop, 1983). *Ras* genes are highly conserved in all eukaryotic cells including yeast. In mammalian cells, the major function of ras proteins is to transduce mitogenic signals, initiated at the cell surface or in the cytoplasm, to the nucleus. In the ten or so years since *ras* oncogenes were first characterized, many details of how ras proteins function in signal transduction in normal and neoplastic mammalian cells have been identified.

A. The GDP/GTP cycle

The ras proteins implicated in human carcinogenesis are part of a large family of proteins known as guanine nucleotide binding proteins or G-proteins (Simon et al., 1991). The activity of ras proteins is dependent upon which guanine nucleotide is bound to the ras protein (review by Santos and Nebreda, 1989). Ras proteins are in their inactive conformation when they are bound by GDP and become activated when GDP is replaced by GTP. The molecules that regulate whether ras is GDP- or GTP-bound thereby determine the activation state of ras proteins.

In quiescent, normal cells, nearly all of the ras proteins are in the inactive GDP-bound form (Haubruck and McCormick, 1991). Upstream

mitogenic signals cause ras to release GDP. Since the majority of free guanine nucleotide in the cytoplasm is GTP, the empty guanine nucleotide binding site of ras proteins quickly become filled by GTP, resulting in the activation of ras. Normal ras proteins have intrinsic GTPase activity, but the intrinsic activity is so low that hydrolysis of bound GTP to GDP by the intrinsic ras GTPase activity alone is very slow (Hall and Self, 1986). However, the ras GTPase activity can be enhanced by proteins known as GTPase activating proteins or GAP proteins (Trahey and McCormick, 1987). When GAP proteins interact with ras, the elevated GTPase activity causes rapid hydrolysis GTP to GDP, and as a result ras becomes inactivated.

Mutations in *ras* genes have been implicated in more than 30% of human malignancies (Barbacid, 1987). The result of activating *ras* gene mutations is that the proportion of GTP-bound ras protein in a cell at any one time is elevated, turning on the ras-mediated signal transduction pathway(s). It is thought that this is the mechanism whereby mutations in *ras* genes contribute to the unregulated growth that is characteristic of cancer cells.

B. GTPase Activating Proteins

Two of GTPase activating proteins (GAPs) have been identified that regulate as proteins in mammalian cells. p120-GAP was the first discovered and is present in all mammalian cells (Trahey and McCormick, 1987). Data indicates that p120-GAP directly regulates ras proteins by facilitating intrinsic ras GTPase activity. When the type 1 neurofibromatosis gene was cloned and sequenced it was found to have considerable homology to the gene encoding p120-GAP (Xu et al., 1990a). Subsequent efforts demonstrated that the NF1-GAP protein, neurofibromin,

also binds to and stimulates the intrinsic GTPase activity of ras proteins (Xu et al., 1990b).

1. p120-GAP

It was initially considered that the difference between mutant and wild type ras proteins was that the activated mutant ras proteins were deficient in their intrinsic GTPase activity and therefore, the level of mutant ras bound to GTP was elevated relative to that of wild type ras. However, *in vitro* studies of mutant and wild type ras proteins did not reveal any significant differences between the GTPase activity of normal and mutant ras (Trahey and McCormick, 1987). These investigators analyzed wild type N-ras, which has a glycine residue at amino acid position 12 (Gly-12 p21), and two mutant N-ras proteins, Asp-12 p21 and Val-12 p21. *Xenopus* oocytes provided a useful *in vivo* assay because of the previous observation that activated (GTP-bound) human ras proteins can induce *Xenopus* oocyte maturation, a change that is easily observed (Birchmeier et al., 1985). Upon injection into the oocytes, the mutant ras proteins induced oocyte maturation much more efficiently than wild type ras. When ^{32}P-labeled mutant or wild type ras proteins were recovered from oocytes, mutant ras proteins remained mostly bound to GTP, whereas wild type ras was bound mainly to GDP. When a non-hydrolyzable analogue of GTP was used in these experiments, the wild type and mutant ras proteins were equally potent inducers of oocyte maturation. Furthermore, when GDP was attached to mutant ras proteins or the wild type ras protein, all were very inefficient inducers of maturation. The ability of both mutant and wild type ras to induce oocyte maturation depended upon whether GDP or GTP was bound to the protein. The results indicated that some factor may be

present in the oocyte milieu that was able to stimulate GTP hydrolysis on wild type ras but not on mutant ras protein.

To investigate whether such a ras-regulating factor might be present in *Xenopus* oocytes, purified wild type or mutant ras proteins were co-injected into oocytes with ^{32}P-phosphate (Trahey and McCormick, 1987). After 3 hours the proteins were extracted and immunoprecipitated with an anti-ras antibody. Following immunoprecipitation, the radiolabeled nucleotides were separated from the ras proteins and analyzed by thin layer chromatography, revealing that wild type ras was almost entirely GDP-bound, whereas mutant ras protein was mainly GTP-bound. A time course demonstrated that the $t_{1/2}$ of GTP bound to wild type ras was 2-3 minutes whereas that of mutant ras was greater than 1000 minutes. The regulating factor participating in the phenomenon, p120-GAP, enhanced the intrinsic GTPase activity of wild type ras proteins but was unable to enhance the GTPase activity of any of the mutant ras proteins used in the assay. p120-GAP was subsequently isolated from oocytes as well as mammalian cells (Trahey and McCormick, 1987) and within a year, the GAP gene was cloned (Trahey et al., 1988).

2. Neurofibromin

Since GTPase-activating proteins regulate ras activity, defects in such proteins could cause elevation of the amount of activated ras in cells and in that way contribute to neoplasia. In other words, normal GAPs may act as tumor suppressors. Evidence to support a role for GAPs as tumor suppressor proteins came from studies of cells derived from tumors that occur at an elevated frequency in type 1 neurofibromatosis. Individuals affected with NF1 develop multiple benign neurofibromas. Neurofibrosarcomas, which occur as a result of malignant transformation of

a neurofibroma, occur at an elevated frequency in NF1 patients (Riccardi and Eichner, 1986). Declue et al. (1992) studied three cell lines derived neurofibrosarcomas that occurred in NFI patients. Immunoprecipitation and Western blot analysis demonstrated that the neurofibrosarcoma cells produce almost no neurofibromin, whereas the control neoplastic (HeLa cells) and nonneoplastic cells (NIH-3T3 cells) produce considerable neurofibromin. The ratio of ras-GTP to ras-GDP was found to be elevated in each of the three neurofibrosarcoma cell lines, similar to the high levels of ras-GTP in v-H-*ras*-transformed NIH-3T3 cells. Since it was possible that *ras* mutations, and not the absence of neurofibromin, was responsible for the high proportion of GTP bound to ras in the neurofibrosarcoma cell lines, they devised a strategy to distinguish whether the ras proteins in each cell line behaved like wild type or mutant ras proteins. Ras proteins were isolated from the three neurofibrosarcoma cell lines and control cell lines and were bound *in vitro* with [α-^{32}P]-GTP, and then were incubated to determine the intrinsic GTPase activity of ras proteins from individual cell lines. The results showed that the ras proteins isolated from v-H-*ras*-transformed NIH-3T3 cells remained greater than 80% GTP-bound, whereas ras proteins from all other cell lines, including the neurofibrosarcoma cell lines, was only 30% GTP-bound. This indicates that ras proteins from neurofibrosarcoma cells retained their intrinsic GTPase activity and the ability to interact with normal GAP proteins in the incubation milieu. When one of these neurofibrosarcoma lines was transfected with a plasmid carrying the catalytic domain of p120-GAP and a selectable marker, the amount of ras in the GTP-bound form in transfectant cells was reduced from 38% to 10% (DeClue et al., 1992). The findings that neurofibrosarcoma-derived ras proteins had normal intrinsic ras-GTPase activity *in vitro*, and that the

amount of ras-GTP in such cells was significantly reduced by the addition of a normal GAP catalytic domain, strongly suggests that the ras proteins in the neurofibrosarcomas were normal and that the elevated level of ras-GTP in neurofibrosarcoma cells was due to deficient GTPase activity.

Comparison of the ratio of ras-GDP verses ras-GTP in sub-confluent and confluent *ras*-transformed NIH 3T3 cells in culture suggests that the activity of GAP proteins is effected by cell density (Zhang et al., 1992). The activity was measured by incubating recombinant ras proteins bound to [α-^{32}P]GTP in buffer containing cell extracts obtained at high or low density. The cell extract obtained from cells at high density had four times the GTPase activity as cell extracts isolated from sub-confluent cells, suggesting that cell-cell contact induces elevation of p120-GAP and/or NF1 (or other GAP proteins) GTPase activity.

One question regarding ras regulation that continues to be explored is the role of neurofibromin and p120-GAP in the regulation of ras proteins in various cell types. Both of these GAPs are expressed in all normal cells that have been examined (Golubic et al., 1992), although neurofibromin is expressed at a ten-fold higher level in brain cells than in cells from kidney, liver, lung, or spleen (Daston et al., 1990). Neurofibromin appears to regulate ras activity in Schwann cells, the cell of origin of neurofibromas and neurofibrosarcomas. It is unclear why p120-GAP does not compensate for the deficiency of neurofibromin GTPase activity in such neurofibrosarcoma cells.

C. Guanine Nucleotide Releasing Factors

The ras-GDP complex is extremely stable in the presence of physiologic concentrations of Mg^{++} *in vitro* (Wolfman and Macara, 1990), and nearly all ras is bound to GDP in normal quiescent cells despite the

relative abundance of GTP in the cytosol (Haubruck and McCormick, 1991). However, ras rapidly releases GDP and becomes GTP-bound in response to specific external stimuli, suggesting the presence of some factor or factors that have the ability to catalyze the release of GDP from the ras-GDP complex.

Guanine nucleotide releasing factors (GRFs) were first identified in non-mammalian systems. The first was the *CDC25* gene product, Cdc25p, of *Saccharomyces cerevisiae* (Jones et al., 1991). Interest in the *CDC25* gene came about as a result of its observed role in the regulation of ras-mediated cyclic AMP activation in yeast (Camonis et al., 1986). In yeast, GTP-bound ras stimulates adenylate cyclase, which is necessary for entrance into the cell cycle, and the *CDC25* gene product is necessary for ras activation (Broek et al., 1987). The *sos* gene of *Drosophila melanogaster* is homologous to yeast *CDC25*, and it also triggers release of GDP from ras (Simon et al., 1991). Because the structure and function of ras is so well conserved among diverse species, it is likely that proteins similar to the *CDC25* product function in mammalian cells to mediate the release of GDP from ras proteins in response to signals that activate ras.

To test for the presence of a factor that promotes the dissociation of GDP from mammalian ras, Wolfman and Macara (1990) incubated ras proteins bound to [α^{32}P]-GDP in rat brain homogenate containing excess unlabeled GDP and Mg^{++}. Under these conditions the half-life of labeled GDP bound to ras was two minutes, compared to more than 60 minutes in the absence of brain cytosol (homogenization buffer only). To ensure that GAP proteins in the homogenate were not the factors responsible for dissociation of GDP, GAP proteins were also added to the homogenization buffer (cytosolic fraction) in a control incubation and were found to have no effect on the release of ^{32}P-labeled GDP from ras. This suggested that

some factor in the homogenate other than GAP proteins caused the release of labeled GDP from ras proteins.

Shou et al. (1992) isolated a mammalian (rat) cDNA encoding a guanine nucleotide releasing factor by using a set of degenerate oligonucleotide primers based on highly conserved regions of the yeast homolog. The rat gene showed approximately 30% homology to yeast and fruit fly guanine nucleotide releasing factor genes. Guanine nucleotide releasing factors have also been identified or isolated from human placenta (Downward et al., 1990) and bovine brain (Huang et al., 1990). It remains unclear whether the guanine nucleotide releasing factors isolated from different types of mammalian cells represent a single protein encoded by homologous genes in various species and cell types, or whether more than one factor is involved. Characterization of this protein or proteins is critical to fully understand ras-mediated signal transduction pathways, particularly the mechanisms(s) whereby ras proteins become activated.

D. Mitogenic Growth Factors

The importance of ras proteins in growth factor induced mitogenesis in mammalian cells was first illustrated by studies in which neutralizing, anti-ras antibodies were micro-injected into quiescent NIH-3T3 cells prior to stimulation by fetal bovine serum. Microinjection of approximately 3 x 10^6 molecules of ras antibodies (0.2% of total cell protein) into the cells, prevented DNA synthesis and subsequent cell division in response to fetal bovine serum stimulation (Mulcahy et al., 1985). Similar amounts of control antibodies had no effect on DNA synthesis and cell proliferation. In addition, the accumulation of GTP-bound ras in rodent fibroblasts

occurred in response to the addition of specific growth factors, such as PDGF (Satoh et al., 1990a) and EGF (Satoh et al., 1990b).

When mitogenic growth factors such as PDGF and EGF bind with the external domain of their respective receptors, the internal domains of the receptors become autophosphorylated on tyrosine residues. The activated internal domains interact with cytoplasmic substrates and phosphorylate tyrosine residues on these substrates (Heldin and Westermark, 1991). Examples of such cytoplasmic substrates include phospholipase C-γ (PLC-γ) (Meisenhelder et al., 1989), p120-GAP (Kaplan et al., 1990; Ellis et al., 1990) and phoshatidylinositol 3-kinase (PI3K) (Coughlin et al., 1989; Kavanaugh et al., 1992). Although GAPs have been implicated in the regulation of ras protein activity, the possible role of PLC-γ or PI3K in ras activation remains to be elucidated.

Elevation of ras-GTP levels as a result of growth factor stimulation of normal cells may occur because phosphorylation of p120-GAP abrogates the ability of p120-GAP to enhance the GTPase activity of ras proteins (Molloy et al., 1992). In non-transformed NIH 3T3 cells, or those transformed by over-expressed c-*ras*, stimulation by PDGF significantly increases the proportion of GTP-bound ras (Molloy et al., 1992). However, when v-*ras*-transformed NIH 3T3 cells are stimulated with PDGF there is little or no increase in the already elevated proportion of ras bound to GTP. One interpretation of these findings is that some *ras* mutations result in ras proteins that are no longer subject to GAP regulation, and as a result do not require growth factor-induced phosphorylation of GAP to increase the amount of activated ras proteins. Alternatively, some types of mutant ras may have constitutionally high guanine nucleotide releasing activity, thereby increasing the amount of ras-GTP.

Much less is know about guanine nucleotide releasing factors (GRFs) than GAP proteins in regard to tyrosine kinase signaling pathways. Work by Zhang et al (1992) suggests that either PDGF or serum induced elevation of GTP-bound ras in NIH 3T3 cells is a consequence of increased GRF activity. Two functionally different c-H-*ras* mutations were employed to arrive at this conclusion. One mutant *ras* gene encodes a protein that has a single amino acid substitution at amino acid 116 (c-H-ras^{116}), the site of guanine nucleotide binding. Ras proteins with this mutation retain their normal sensitivity to regulation by p120-GAP and NFI, but have an 10-fold increase in the intrinsic guanine nucleotide exchange rate (Der et al., 1988). With c-H-ras^{116} transfectants, if the serum/PDGF-induced elevation of ras-GTP depends on inactivating p120-GAP or NFI, the addition of serum or PDGF should increase the level of ras-GTP. Conversely, if serum/PDGF-induced increase in GTP-bound ras is dependent upon activation of a GRF, then serum or PDGF stimulation should have no effect on the level of ras-GTP. When confluent NIH 3T3 cells transfected with c-H-ras^{116} were treated with serum or PDGF, the PDGF receptors were activated, yet there was no increase in the level of GTP-bound Ras. To verify that the effect of serum is to stimulate GRF activity, another ras protein mutated at amino acid 36 was used in similar experiments. Mutant c-H-ras^{36} has about the same transforming ability as c-H-ras^{116} as judged by the frequency of foci formed in NIH 3T3 cells and the level of GTP bound to ras in each of the transfected cell strains. However, in contrast to NIH 3T3 cells transfected c-H-ras^{116}, those transfected with c-H-ras^{36} are resistant to regulation by p120-GAP and neurofibromin, and the cells release GDP at the same rate as cells with non-mutated ras. When confluent NIH 3T3-ras^{36} transfectants in serum free conditions were stimulated with serum or PDGF, there was a three to four-fold increase in

the level of GTP bound to ras. Since the c-H-*ras*[36] protein is not responsive to GAP or neurofibromin regulation, the increase in GTP-bound ras was very likely the result of growth factor mediated stimulation of guanine nucleotide releasing activity. This suggests that the mechanism whereby ras becomes GTP-bound in response to serum or growth factor stimulation at least partially involves stimulation of guanine nucleotide releasing factor activity. Recent investigations have shown that activation of the epidermal growth factor receptor in mammalian cells also leads to activation of guanine nucleotide releasing factor, leading to subsequent activation of ras proteins (Gale et al., 1993; Buday and Downward, 1993).

A combination of GTPase activity and guanine nucleotide releasing activity is responsible for ras protein regulation. Oncogenic synergy could occur as a consequence of elevated growth factor expression and the loss of normal GAP protein activity. Endogenous production of mitogenic growth factors increases the formation of GTP-ras by promoting the release of GDP from ras proteins. Loss of GTPase activity results in decreased hydrolysis of GTP-ras. The overall effect of these combined changes would be to increase the fraction of ras that is bound to GTP. This observation demonstrates both oncogenic synergy and the importance of mitogenic signal transduction to maintaining normal cell growth.

V. Evidence of Autocrine Mechanisms in Transformation: The Role of PDGF-B in Neoplasia

A. The Transforming Gene of SSV is Homologous to the c-*sis* (PDGF-B) Gene

By the early 1980's investigators had identified several oncogenes and were attempting to discover the mechanisms whereby each of these accomplished its neoplastic effect. Sporn and Todaro (1980) demonstrated that when grown in agar, normal cells grow like tumor cells if a fraction of the spent medium from tumor cells is added to the normal cells. From one of these tumor cell lines, which was derived from a human osteosarcoma, a PDGF-like growth factor was isolated (Heldin et al, 1980). Based on these facts, Sporn and Todaro (1980) purposed the autocrine hypothesis of carcinogenesis, namely that cancer cells produce a mitogenic growth factor(s) and the appropriate receptor(s) for the growth factor, resulting in continuous growth and division of such cells. Although it appeared that growth factors might play a causal role in the development of human and animal cancers, there was no evidence to indicate a direct relationship between any known transforming gene and a growth factor.

Support for the autocrine hypothesis came from different groups of scientists who were investigating what appeared to be unrelated questions. First, the transforming gene of the simian sarcoma virus (SSV), was identified and isolated from a sarcoma that occurred in a woolly monkey, and was sequenced by investigators at the National Cancer Institute (Devare et al., 1983). About three months later, Antoniades and Hunkapiller (1983) reported the partial amino acid sequence of PDGF. When they published their work they were unaware of the homology between the protein sequence of PDGF and that of the SSV product. Less than two months after the sequence of PDGF was published, a protein database was used to demonstrate the sequence homology between the amino acid sequence of PDGF and the SSV encoded protein. (Waterfield et al., 1983; Doolittle et al., 1983). This was the first direct evidence that a growth factor gene could act as a transforming oncogene.

B. Structure and Function of PDGF-B and Its Receptor

PDGF isolated from human platelets consists of two distinct but related polypeptides designated the PDGF-A chain and the PDGF-B chain. The active protein is a dimer including either of the homodimers (PDGF-AA and PDGF-BB) or the heterodimer (PDGF-AB) (reviewed by Hannink and Donoghue, 1989). The human PDGF-A chain (Betsholtz et al., 1986) and PDGF-B chain (Swan et al., 1982) are encoded on chromosomes 7 and 22 respectively, and are approximately 50% homologous. The viral and normal vertebrate genes that encode PDGF-B are known as v-*sis* and c-*sis*, respectively.

Although their sequences are nearly identical, there are significant differences between the mature c-*sis* and the v-*sis* mRNAs. Mature c-*sis* mRNA includes unusually long 5' and 3' untranslated sequences (Chiu et al., 1984). Studies in which the 5' untranslated sequence of c-*sis* was deleted demonstrated that the 5'-untranslated sequence exerts a negative regulatory effect on the translation efficiency of the mature c-*sis* transcript (Rao et al., 1988; Fen and Daniel, 1991; Franklin et al., 1991). The SSV-derived v-*sis* gene is under virally encoded transcriptional and translational control and lacks the 3' and 5' untranslated sequences that inhibit translation efficiency of c-*sis* mRNA. Comparison of v-*sis* and c-*sis* coding regions has shown that there are only a few amino acid differences (Chiu et a., 1984). Since the c-*sis* gene of the woolly monkey, the animal from which SSV originated, has not been sequenced it is not known whether the differences between the coding regions of v-*sis* and c-*sis* are mutations that contribute to the transforming activity of v-*sis* or whether they are due to species specific differences between the woolly monkey and the human c-*sis* gene. When the c-*sis* coding sequence was inserted into the genome of a non-transforming

retrovirus, the recombinant retrovirus was able to transform cells that subsequently formed sarcomas when injected into mice (Pech et al., 1989), suggesting that the differences in the coding sequences between v-*sis* and c-*sis* are unimportant, and that high levels of v-*sis* or c-*sis* gene products generated by viral vectors account for the transforming ability of *sis* genes.

The PDGF protein initiates mitogenesis by binding to cell surface receptors which leads to dimerization of the receptors. There are two different PDGF receptor peptides, known as the PDGF-α and PDGF-ß receptors, which form receptor dimers when they are bound by PDGF (Yarden et al., 1986; Gronwald et al., 1988) There are three possible receptor dimer combinations including αα, αß and ßß. The PDGF-AA growth factor dimer can bind only to the PDGF-αα receptor, whereas PDGF-AB can bind to either PDGF-αα or PDGF-αß. PDGF-BB can bind to any of the receptor dimers, but binds to the PDGF-ßß receptor dimer with significantly higher affinity than either PDGF-AB and PDGF-AA dimers (Heldin, 1992). Each of the receptor dimers (any combination) can transduce mitogenic signals, but only receptor dimers that include the PDGF-ß receptor peptide participate in chemotaxis and actin organization (Nister et al., 1988; Eriksson et al., 1992). Furthermore, the PDGF-B/c-*sis* protooncogene is much more efficient than PDGF-A oncogene as a transforming gene in animal and human cells (Bywater et al, 1988; Beckmann et al., 1988). The full significance of the various types of platelet-derived growth factors and receptor dimers and their interactions is still not completely understood.

C. The Role of PDGF-B Expression in Neoplasia

Following the discovery of near complete sequence homology between v-*sis* and the human c-*sis* gene, investigators sought to understand the

mechanism whereby the v-*sis* oncogene transforms cells, and to determine whether such a mechanism is activated in human tumor cells that express PDGF-B/c-*sis*. The simplest assumption was that production and secretion of PDGF by cells that express the PDGF receptor resulted in continuous receptor activation, and therefore continuous transduction of downstream mitogenic events, including cell division. The following sections discuss the role of PDGF-B expression in neoplastic cells generated in culture and those which have been derived from benign and malignant human tumors.

1. Autocrine Activation of the PDGF-receptor

In response to PDGF stimulation, PDGF receptors become phosphorylated on cytoplasmic tyrosine residues (reviewed by Heldin, 1992). Anti-phosphotyrosine antibodies have therefore been useful in detecting activation of PDGF receptors in immunoblot assays (Ek and Heldin, 1984; Zippel at al., 1986). When NIH-3T3 cells or normal human fibroblasts in culture are placed in medium containing no exogenous growth factors, PDGF receptors remain present on the cell surface and are not phosphorylated on tyrosine residues (Ross et al, 1986). Under similar conditions, PDGF-ß receptors of SSV-transformed NIH-3T3 cells and SSV-transformed human fibroblasts are down-regulated (i.e., are not present on the cell surface due to internalization) and constitutively phosphorylated on tyrosine residues (Huang and Huang, 1988; Fleming et al., 1992b; Keating and Williams, 1988). When exogenous PDGF or serum is added to quiescent, normal human fibroblasts or NIH-3T3 cells in culture, their PDGF receptors become tyrosine phosphorylated and down-regulated like the receptors of SSV/v-*sis* transformed cells (Ross et al., 1986). Since PDGF-ß receptors are constitutively phosphorylated in SSV/v-*sis* transformed cells, and the phosphorylation of PDGF-ß receptors is associated with

PDGF-B-induced cell division in normal cells, SSV/v-*sis* could bring about transformation of cells by continuously producing PDGF-B proteins, and in that way activating PDGF-ß receptors by continuous tyrosine phosphorylation.

When PDGF is added to quiescent, normal fibroblasts in serum free culture conditions they enter into cell division (Heldin et al., 1981; Johnsson et al, 1985). But when anti-PDGF neutralizing antibodies are added to the quiescent fibroblasts in addition to the exogenous PDGF, the PDGF-induced growth of normal fibroblasts is prevented. If SSV/v-*sis* transforms cells solely through an autocrine mechanism by secreting PDGF-B and activating PDGF receptors extracellularly, then neutralizing antibodies should inhibit the effects of autocrine activation as they do for normal cells. When neutralizing anti-PDGF antibodies were used to inhibit the growth of SSV transformed cells in serum free medium, only partial inhibition (Johnsson et al., 1985) or no inhibition (Huang et al., 1984; Huang and Huang, 1988) was achieved. These findings suggest either that the autocrine interaction of PDGF with its receptor occurs in an internal compartment that is unaccessible to neutralizing antibodies, or that some other mechanism, other than external receptor activation, is responsible for the ability of SSV transformed cells to grow in medium lacking exogenous growth factors.

2. Experiments with Suramin

To examine whether autocrine activation involving PDGF and the PDGF receptor occurs in an internal compartment, investigators used the antifilarial drug, suramin, which inhibits interactions between PDGF and

the PDGF receptor (Williams et al., 1984; Garrett et al., 1984), as well as interactions between other growth factors and their receptors (Minniti et al., 1992, Sato and Rifkin, 1988; Betsholtz et al., 1986; Mills et al., 1990). Although suramin has been reported to affect various enzymes such as DNA polymerase (Jindal et al., 1990) and reverse transcriptase (Levine et al, 1986), the effects of suramin at lower concentrations (up to 100μg/ml) appear to be relatively specific for interferring with growth factor/growth factor receptor interactions in culture systems using cell lines such as NIH-3T3, MSU-1.1, and strains derived from these cell lines.

Concentrations of 50-100μg/ml of suramin inhibit the transformed phenotype of SSV transformed NIH-3T3 cells in culture (Garrett et al., 1984, Betsholtz et al., 1986) and reverse the down regulation of the PDGF-receptor in such cells. Within a few minutes of suramin treatment, PDGF receptors in SSV-transformed NIH 3T3 cells are no longer tyrosine phosphorylated (Huang and Huang, 1988). However, when NIH 3T3 cells are transformed by oncogenes that are not dependent upon growth factor activation, suramin has no effect. For example, suramin has no inhibitory effect on *erb*B-2-transformed NIH-3T3 cells (Fleming et al., 1989). Whether suramin exerts its inhibitory effect on the cell surface and/or in the cytoplasm is not known. However, it is likely that suramin can inhibit growth factor interactions both intracellularly and on the cell surface. Like neutralizing antibodies, suramin inhibits the proliferative effects that TGF-α and PDGF-B exert on normal fibroblasts in culture. Furthermore, suramin, unlike neutralizing antibodies against growth factors, completely inhibits the growth of SSV/v-*sis* transformed cells in culture (Huang and Huang, 1988; Keating and Williams, 1988).

Such observations have been validated in the MSU-1.1 lineage developed in this laboratory by Morgan et al. (1991). When the

immortalized, non-neoplastic human fibroblast line, MSU-1.1, was transfected with a plasmid carrying the v-*sis* gene, transformants formed large colonies in soft agar and grew rapidly in serum free medium. The addition of 50μg/ml suramin to the experimental conditions completely prevented the MSU-1.1-v-*sis* cells from forming colonies in soft agar and from proliferating in serum free medium (Yang et al., 1994). If the effect of suramin is to interrupt growth factor/receptor interactions, oncogenes such as H-*ras* and *erb*B-2, which act downstream of growth factor initiated signalling pathways, would not be expected to be affected by suramin. When serum free growth and anchorage independence of MSU-1.1-v-H-*ras* cells were studied, they were unaffected by levels of up to 100μg/ml suramin (Yang et al., 1994). This data supports the hypothesis that SSV/v-*sis*-transformed cells depend upon PDGF activation of PDGF receptors in an autocrine manner to attain the transformed phenotype. Because suramin completely inhibits growth of SSV/v-*sis* transformed cells and anti-PDGF antisera causes slight or no inhibition of such cells, PDGF-B appears to have the capability to activate the PDGF receptors through an internal autocrine mechanism (Keating and Williams, 1988).

3. PDGF-B Expression in Human Tumor Cells

There are several reports describing the co-expression of PDGF-B and the PDGF-β receptor in fixed human tumor tissue and in human tumor-derived cell lines (for review see Silver, 1992). Some of the types of human neoplasms that commonly express PDGF-B and its receptor include glial cell tumors (Maxwell, 1990; Fleming et al., 1992a; Hermanson, 1992), tumors that arise in mesenchymal tissues (Richter and Graves, 1988; Fleming et al., 1992b; Malik et al., 1991), gastric carcinomas (Chung and Antoniades,

1992), and lung carcinomas (Antoniades et al., 1992). These studies suggest that autocrine mechanisms involving PDGF-B have a causal role in the formation of various tumors, but do not directly proove that an autocrine mechanism contributes to the neoplastic phenotype.

Heldin and Westermark and their colleagues studied a human osteosarcoma cell line and attempted to demonstrate the presence of autocrine activation involving PDGF. This cell line, known as U-2 OS, expresses PDGF-B and its receptor (Betsholtz et al., 1984). Efforts to abrogate *in vitro* features of transformation exhibited by cell line U-2 OS (and other human tumor cell lines) using receptor antibodies or suramin have failed to show any inhibitory effect (Heldin and Westermark, 1991). For example, suramin reversed PDGF receptor down regulation in U-2 OS cells, but caused no inhibition of the DNA synthesis associated with cell division (Richter and Graves, 1988). Since suramin was shown to significantly inhibit DNA synthesis of SSV/v-*sis* transformed cells in culture (Fleming et al., 1989), induction of DNA synthesis and subsequent cell division in U-2 OS cells in serum free medium is not dependent upon an autocrine mechanism involving PDGF-B and its receptor. Suramin also does not stop the serum free growth of several established human fibrosarcoma cell lines, such as HT-1080 (Dave Reinhold, unpublished results) that have previously been shown to express PDGF-B mRNA and protein (Pantazis et al., 1985). However, one recent report (Fleming et al., 1992b) showed that the growth of other human tumor cell lines are adversely affected by suramin. In this study 100 μg/ml of suramin inhibited the serum free growth of a number of human sarcoma and glial cell tumor lines to various degrees. Because suramin is not a specific inhibitor of PDGF-B, it is possible that other activated growth factor pathways in these tumors are being inhibited by suramin. Therefore, there

is still no conclusive, direct evidence that PDGF-B has any effect on the neoplastic phenotype of any cell line derived from a human tumor.

4. Paracrine Mechanisms in Neoplasia

Failure to demonstrate that PDGF-B expression had a role in any feature of transformation in human tumor-derived cells led to the speculation that PDGF-B expression might be is an early change in multistep carcinogenesis. For example, PDGF-B expression might fulfill a role similar to that of TPA in mouse skin carcinogenesis, namely, by causing proliferation of an abnormal cell, thereby providing a much larger population of cells, which are the target for further oncogenic changes. In such a scenario it is possible that additional oncogenic changes eventually render the production of PDGF-B unnecessary for the malignant phenotype. Another possibility is that PDGF-B secreted by tumor cells acts on non-neoplastic cells in the tumor environment. Some human cancer cells produce PDGF-B but have no PDGF receptors (Rozengurt et al., 1985; Westermark et al., 1986; Peres et al., 1987), suggesting that *paracrine* effects of growth factors, i.e., contribute to neoplasia. Using immunohistochemical methods and *in situ* hybridization, Heldin and Westermark and their colleagues showed that non-neoplastic cells in the tumor environment, including hyperplastic stromal cells and vascular endothelial cells, express high levels of PDGF-B receptor mRNA and protein, whereas the PDGF-B expressing neoplastic cells express low levels of PDGF-B receptor protein and mRNA (Hermanson et al., 1992). Similar studies showed that AIDS-related Kaposi sarcomas consist of two populations of cells, a minority population of cells that express PDGF-B and the PDGF-B receptor, and a majority population that express only the PDGF-B receptor (Sturzl et al., 1992). The cells that express only PDGF-B

receptors appeared to be normal fibroblasts and/or smooth muscle cells rather than neoplastic cells. These observations support the hypothesis that neoplastic cells use normal cells from the tumor environment for some undefined role in neoplasia.

PDGF-B is a strong chemo-attractant for fibroblasts (Seppa et al., 1982) and endothelial cells (Bar et al., 1989). Proliferation of fibroblasts and endothelial cells is required for *stroma formation*, which is the formation of connective tissue and new blood vessels (neovascularization) in the tumor environment. The ability to stimulate neovascularization is a characteristic of malignant tumors and is considered necessary to provide nutrients for rapidly growing tumors. To examine whether the expression of PDGF-B contributes to stroma and blood vessel proliferation *in vivo*, Forsberg et al (1993) transfected human WM9 melanoma cells, which do not express PDGF-B, with a plasmid carrying the coding sequence of human PDGF-B and a neomycin resistance gene. Control cells were injected with a construct that differed from the experimental construct in that it lacked the PDGF-B gene. PDGF-B- expressing transfectants and control transfectants were injected into five athymic mice each. All of the mice developed progressively growing malignant tumors. The PDGF expressing cells produced one cubic centimeter tumors in five weeks. In contrast, control-transfected cells required two additional weeks to reach an equivalent size (not considered to be significant by the authors). Examination of histological sections of the tumors demonstrated a dramatic difference in stromal formation. PDGF-B expressing cells formed a tumor with a well developed network of connective tissue and blood vessels surrounding and penetrating the tumors. In addition, the vasculature and connective tissue of the PDGF-B producing cells were organized in nodular, connective tissue encapsulated

regions. Mock-transfected cells and untreated WM9 cells produced tumors that lacked substantial connective tissue and blood supply. Furthermore, large areas of necrosis were present in tumors produced by mock-transfected or non-transfected WM9 cells. Similar areas of necrosis were not found in the tumors formed by the PDGF-B transfected cells. Tissue necrosis presumably results from a blood supply that is insufficient to meet the nutritional and waste disposing demands of highly metabolic cancer cells. The results of these experiments indicate that PDGF-B can participate in stroma formation in human cells *in vivo*.

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CHAPTER II

Evidence that Formation of Neurofibrosarcomas in Individuals with Type 1 Neurofibromatosis is Associated with Activation of Growth Factor Pathways

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ABSTRACT

One of the features of Type 1 neurofibromatosis, a dominantly inherited syndrome, is that affected individuals commonly form numerous benign neurofibromas, which arise from Schwann cells that surround peripheral nerves. Rarely, a neurofibroma cell undergoes malignant transformation giving rise to a neurofibrosarcoma. Although neurofibrosarcomas are rare even in NF1 patients, they are much more common in such individuals than in the general population. We used a reverse transcription-polymerase chain reaction (RT-PCR) assay to examine whether tumor cell lines derived from NF1-associated neurofibromas and neurofibrosarcomas expressed TGF-α and PDGF-B mRNA. Five of six neurofibrosarcoma cells expressed high levels of TGF-α and PDGF-B mRNA similar to the levels seen in human fibrosarcoma-derived cells. Neurofibroma cells also express PDGF-B mRNA but at a lower level than that of neurofibrosarcoma cells. Since normal rat Schwann cells and human Schwann cells derived from neurofibromas do not express receptors for serum growth factors such as PDGF, we tested whether the neurofibrosarcoma cells in culture proliferate in response to PDGF, TGF-α and bFGF. Our results indicated that two of the three neurofibrosarcoma cell lines tested respond to one or more of these growth factors. The third, which expresses high levels of PDGF and TGF-α, grows extremely rapidly in serum-free medium and is not inhibited by suramin. We also demonstrate that one of two neurofibrosarcoma cell lines capable of growth in medium lacking

exogenous growth factors is completely inhibited from growth in such conditions in the presence of 50 μg/ml of suramin. Our results support a role for activation of growth factor pathways including elevated expression of growth factors and growth factor receptors in the formation of neurofibrosarcomas that occurs in individuals with NF1.

INTRODUCTION

Type I neurofibromatosis (NF1) is an autosomal dominantly inherited disease that affects approximately one in 3000 individuals and is characterized by multiple neurofibromas (benign tumors), cafe' au lait spots, lisch nodules, bony dysplasia, learning disabilities, and a significant predisposition to the development of malignant tumors (1). Neurofibromas arise from Schwann cells, which form the sheaths around peripheral nerves (2). The presence of numerous neurofibromas in affected individuals suggests that very few changes, or possibly only a single change, in addition to the constitutional NF1 defect, are required to cause a Schwann cell to proliferate and form a neurofibroma. The change (or changes) that causes the development of neurofibromas in affected individuals is unknown.

Neurofibrosarcomas, also known as malignant schwannomas or malignant peripheral nerve sheath tumors, occur as a consequence of malignant transformation of a neurofibroma cell and are the most common of the malignancies that occur at an elevated frequency in NF1 patients (2). Although only a minority of NF1 patients develop neurofibrosarcomas, the frequency of these malignancies is significantly higher in affected individuals than in the general population. In fact, more than half of all neurofibrosarcomas that are diagnosed occur in NF1 patients, even though such individuals compose only a small segment of the population

(3). Nevertheless, the chance of a neurofibroma giving rise to a neurofibrosarcoma is relatively small, suggesting that multiple changes are required for this to occur.

The gene that is constitutively defective in individuals with NF1 has been cloned and sequenced (4), and the gene product has been termed neurofibromin. Analysis of the NF1 coding sequence revealed that one segment of the gene encodes a domain homologous to the catalytic domain of the GTPase-activating protein, p120-GAP (5). GTPase-activating proteins enhance the intrinsic GTPase activity of ras proteins, and therefore favor hydrolysis of active GTP-ras to inactive GDP-ras (6). In normal quiescent cells, the majority of ras is in the inactive GDP-bound form. However, ras rapidly becomes activated in response to signals initiated by mitogenic growth factors such as PDGF-B (7). The proteins involved in mediating this growth factor-initiated signal are guanine nucleotide releasing factors (8, 9), which stimulate the release of GDP from GDP-ras. Since the majority of free guanine nucleotide in the cytosol is GTP, ras not bound by a guanine nucleotide quickly becomes activated by binding to GTP. The finding that the NF1 gene, one allele of which constitutively is defective in NF1 patients, encodes a protein with a domain homologous to p120-GAP, suggested that defects in an allele of this gene predisposes affected individuals to forming neurofibromas because of a deficiency in inactivation of ras. If so, NF1 would represent a tumor suppressor gene and the cells in neurofibrosarcomas can be expected to have defects in both alleles of the NF1 gene. Evidence that this is the case, and that neurofibromin functions as a GTPase-activating protein, comes from study by DeClue et al. (10). They demonstrated that human neurofibrosarcoma cell lines from NF1 patients have normal ras proteins, but are virtually

devoid of neurofibromin and have high levels of ras-GTP, as expected if the cells lack normal regulation of ras protein activity.

As noted above, growth factors stimulate the release of GDP from ras, resulting in binding of ras to GTP and therefore to ras activation. If a cell carrying a mutation in one or both alleles of the NF1 gene were to upregulate expression of a growth factor gene, such as the gene encoding PDGF, this would enhance the effect of their decreased GTPase activity by increasing the level of ras in the activated form, i.e., GTP-ras. It has been shown that extracts from neurofibromas from NF1 individuals contain mitogenic activity (11, 12) and that cells from neurofibromas produce angiogenic factors (13). Several growth factors, including PDGF, could account for these activities. Certain growth factors can stimulate the growth of rat Schwann cells (14) and human neurofibroma-derived schwann cells (12) in standard cell culture. Furthermore, addition of cyclic AMP or exogenous stimulation of the production of this molecule has been shown to cause rat Schwann cells to respond to additional growth factors (15, 16). The question of the status of growth factor expression by cells from neurofibrosarcomas and its possible effect on the growth of such cells has not been examined. To determine how neurofibrosarcomas differ from neurofibromas, we have compared cells which we derived from such tumors, taken from individuals with NF1, for their expression of growth factors, for the effect of growth factors on the proliferation of such cells, and for evidence of dependence of the cells on such growth factors.

Materials and methods

Culturing tumor-derived cells

The tumor material was fragmented into smaller pieces and mechanically agitated using a sterile syringe and cannula to free individual cells from the tumor tissue. Some of the resulting cells and small tumor fragments were placed in 75 cm^2 tissue culture flasks (Corning Glass Works, Corning N.Y.) and cultured in Eagle's minimum essential medium supplemented with 0.2 mM aspartic acid, 1.0 mM sodium pyruvate, 0.2 mM serine, penicillin (100 U/ml), streptomycin (100 μg/ml), hydrocortisone (10 μg/ml), and 5% and/or 10% fetal bovine serum (culture medium). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and air.

Assay for tumorigenicity

Cells (1×10^6 to 10×10^6) in exponential growth were injected subcutaneously in the scapular region of six week old BALB/c athymic mice. Alternatively, small pieces of tumor tissue were implanted subcutaneously into these animals. The mice were examined weekly for tumors. Tumors that resulted were excised, fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin prior to histological evaluation.

RNA isolation

Cells were grown in 150 mm-diameter dishes in culture medium containing supplemented calf serum rather than fetal bovine serum. Total RNA was isolated from cells just prior to their reaching confluence, using the procedure of Chomczynski and Sacchi (17) essentially as described. The RNA was resuspended in diethylpyrocarbonate-treated H_2O, and stored at -80°C. The concentration of RNA was determined from the optical density at 260nm, and the integrity of the RNA was qualitatively determined from the appearance of ribosomal RNA bands on 1% agarose/formaldehyde gels.

Determination of the level of expression of mRNA

Synthesis of cDNA using Moloney murine leukemia virus reverse transcriptase (New England Biolabs) and amplification of specific cDNAs using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), RT-PCR, was carried out using the procedure described by Yang et al. (18) with the following modifications. One microgram of total RNA was added to a series of 500 μl Eppendorf tubes, and an equal volume of triple distilled water was added to an additional tube to be used as a negative control. Random hexamer primers (0.1 mg/ml, Pharmacia/LBK, Piscataway, NJ) were substituted for oligo $(dT)_{12-18}$, and 20 units/ml RT were used instead of 2.5 units/ml. Five μl of reverse transcriptase cocktail were added to each tube, and the samples were incubated at 37°C for 1 hour. A Taq polymerase cocktail was made containing 2.22 mM $MgCl_2$, 66.67 mM KCl, 16.67 mM Tris-HCL (pH 8.55), 0.44 mM deoxynucleotide triphosphate, 1.11 ng/μl of each primer, and 0.055 units/μl of Taq polymerase and the specified sets of primers. Once the RT assay was completed, 45 μl of the Taq cocktail was added to each tube followed by 100 μl of paraffin oil. The tubes were then placed in the DNA thermal cycler (Perkin-Elmer Cetus), using an

initial 5 min denaturation step at 94°C, followed by up to 30 cycles of denaturation for 1 min at 94°C, annealing at 50°C for 1 min, and polymerization at 72°C for 2 min. After 30 cycles, a 7 min extension at 72°C was performed. The primers used for PDGF-B were those of Wang et al. (19); those for TGF-α were chosen by us using the published sequence (20). They are located in separate exons to eliminate amplification of the genomic DNA of that gene. The PCR primers were synthesized in the Macromolecular Structure Facility at Michigan State University. The GAPDH primers were used as an internal control to insure that amplification had occurred equally in each reaction tube as described (21).

Assay for growth factor independence

Cells growing in culture medium were trypsinized, counted, and plated in culture medium containing only 2% fetal bovine serum into 96-well microtiter plates (Corning Glass Works, Corning, NY) at a density of 500 to 3000 cells/microwell (depending upon plating efficiency and rate of growth of individual cell lines). After 24 h of incubation, the medium was replaced with McM medium (18) modified to contain only 0.1 mM calcium, rather than the usual 1.0 mM calcium, and containing the serum replacement supplements specified by Ryan et al. (22) but lacking any exogenous protein growth factors (designated SR_2). In some instances suramin was added. It was obtained as a sodium salt in lyophized form (FBA, Germany, distributed by Miles Inc., West Haven, CT) and used at the designated concentration. The cells were fed with the appropriate medium every two to three days.

To determine the amount of cell proliferation that occurred in each microwell, we used a soluble tetrazolium/formazan solution to determine the relative number of cells in each microwell from the change in

absorbance, essentially as described (23, 24). Briefly, a 1 mg/ml solution of 2-3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) was made in McM medium containing only 0.1 mM calcium and with 10 mM Hepes (pH 7.2) to maintain the pH, and lacking phenol red. A 1.53 mg/ml solution of phenazine methosulfate (PMS) was made in phosphate buffered saline and was added to the XTT solution to obtain a 0.05 mM solution of PMS in 1 mg/ml XTT (150 μl of 1.53 mg/ml PMS per 6 ml of 1 mg/ml XTT). The medium was removed from the microwells and replaced with 200 μl of this modified McM medium and 50 μl of the PMS/XTT solution. The color reaction was allowed to proceed for 4 h at 37C° in a humidified atmosphere of 5% CO_2 and air. After the 4 h incubation, the microwell plate was placed on a shaker for 30 min and then the absorbance of each well was determined with a Zmax Precision Microplate Reader (Molecular Devices Corp., Menlo Park CA).

Assay of cellular response to specific growth factors

Cells growing in culture medium were trypsinized and plated at 5 X 10^4 to 75 X 10^4 cells per 60 mm-diameter culture dish in 5 ml of culture medium with 5% fetal bovine serum. After 24 h, the medium was removed and replaced with 4 ml of McM medium containing only 0.1 mM calcium and SR_2. After another 24 h, the medium was changed to the specified medium The medium was changed on day 3 and cell growth was determined on day 6 by counting cells in triplicate 60 mm culture dishes.

Results

Establishing cell lines from tumors from NF1 patients

To investigate the possible role of protein growth factors in the neoplastic transformation of human Schwann cells into neurofibromas and neurofibrosarcomas, it was important to derive cell lines from fresh tumors at the time of surgical resection in order to have relatively early passage cells for comparative studies. A total of twelve neurofibromas were obtained. Eleven were surgically removed from an NF1 patient with multiple benign tumors and provided to us by Dr. William Mercer of Michigan Capital Medical Center, Lansing, MI and the twelfth was obtained from another NF1 patient and provided to us by Dr. Mark Lebwohl, a dermatological surgeon, from New York, NY. The tumor tissue was transported in Lebovitz medium containing 100 μg/ml Gentamicin sulfate. Upon arrival, approximately one third of the tissue was immediately fixed in 10% formalin for histopathological analysis. The rest was minced, and the cells were separated as described in the materials and methods section. Half of the remaining material was cryopreserved at -135°C or lower for future studies; the rest was placed into culture as described in the methods section. The time between removal from the patient and introduction into cell culture was always less than 20 h. The neurofibroma cell lines derived from the first NF1 patient were designated NFb-1 through Nfb-11; that from the second NF1 patient was designated NFb-12.

Three neurofibrosarcomas from unrelated NF1 patients were similarly obtained from surgeons through our colleague Dr. Mark Zalupski of the Division of Hematology Oncology, Wayne State University School of Medicine, Detroit, MI. These three tumors were handled in the same way as

the neurofibroma tissue. The cell lines derived from these three patients are referred to as NF1-WSU-4, NF1-WSU-10, and NF1-WSU-17.

Verification of the presence of neoplastic cells in the tumor-derived cell population

Neurofibromas

The histopathology report from the pathologist at Michigan State University on the material we fixed in formalin from the original twelve human neurofibromas confirmed the diagnosis that the tumors were neurofibromas. We tested tumor fragments that had been placed into cell culture, as well as early passage cells in culture, for tumorigenicity in athymic mice using the techniques described in the materials and methods section. In virtually every case, small nodules developed at the site of injection or implantation after one month. If not removed, the majority persisted for several months, but eventually regressed. The nodules that were removed were fixed and stained. Every single tumor analyzed demonstrated a histological pattern consistent with the diagnosis of a human neurofibroma (Figure 1). These results verify that a significant percentage of the early passage neurofibroma cells that we used in the studies of the expression of growth factors to be described below were composed of neoplastic Schwann cells. This finding is significant because it has reported that neoplastic Schwann cells in neurofibroma-derived cell populations rapidly become overgrown by fibroblasts.

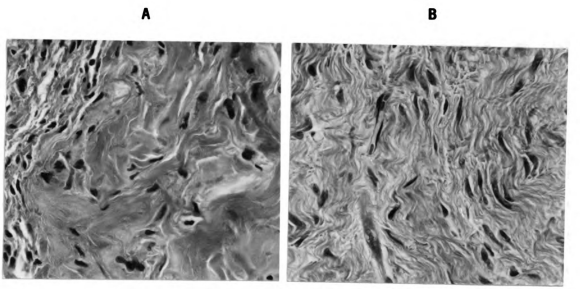


Figure 1. Hematoxylin and eosin stained sections of neurofibromas.

(A) Neurofibroma NFb-10 that occurred in patient with Type 1 neurofibromatosis. (B) Neurofibroma that occurred when neurofibroma cell line NFb-10 was injected subcutaneously into an athymic mouse.

Neurofibrosarcomas

Similar studies were carried out with early passage populations of the cell lines derived from the three neurofibrosarcomas obtained from Wayne State University. The cells from these three tumors gave rise to vigorously growing cell lines that have continued to replicate indefinitely (i.e., >100 population doublings). Therefore, we also examined the tumorigenicity of later passage cells from each of these three cell lines. Cells from both the early passage and the late passage populations from two of the cell lines, i.e., NF1-WSU-10 and NF1-WSU-17 produced progressively-growing tumors at the site of injection with a relatively short latency (see Table I). The histopathology report from the pathologists at Michigan State University on the original tissue that we fixed in formalin on the day the resected tumors were received agreed with the pathology reports subsequently received from Wayne State University indicating that all three of the tumors were neurofibrosarcomas. The histopathology of the tumors that developed in the athymic mice after injection of the cell lines referred to above indicated that they were neurofibrosarcomas. These tumors strikingly resembled the histopathology of the original human tumors from which they were derived (see Figure 2).

For the sake of comparison, three additional neurofibrosarcoma cell lines were obtained from Dr. Thomas Glover from the Department of Pediatrics of the University of Michigan. One, designated NF1-90.8, was from one NF1 patient; the other two, designated NF1 88.3 and 88.14, are cell lines derived from a primary and a recurrent neurofibrosarcoma from

Table I. Tumorigenicity of neurofibrosarcoma-derived cell lines

Cell line	Frequency of Tumors	[a]Latency
NF1-WSU-4	0/4	NA[b]
NF1-WSU-10	4/4	5
NF1-WSU-17	4/7	36
NF1-90.8	6/8[c]	18
NF1-88.3	0/2[d]	NA
NF1-88.14	0/2[d]	NA

[a]Number of weeks for tumor to reach 0.6 cm in diameter.

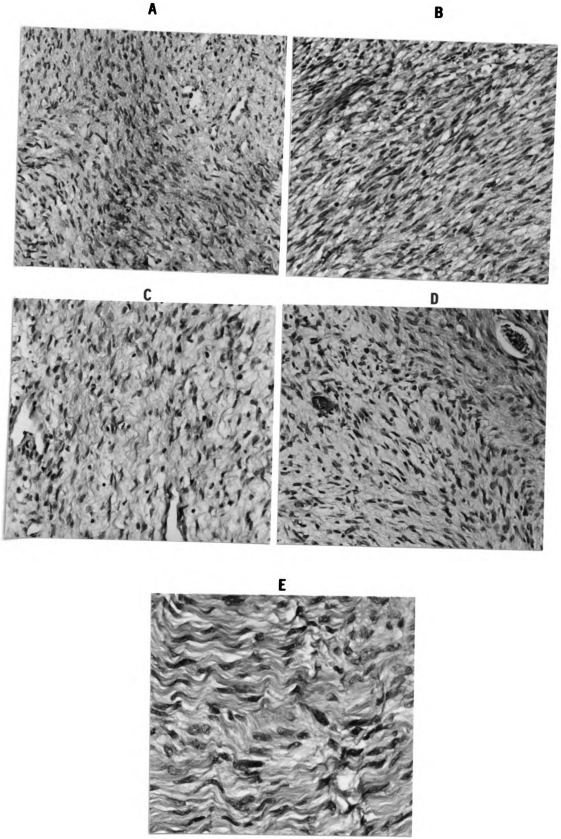
[b]NA, not applicable.

[c]Original neurofibrosarcoma from which cell line NF1-90.8 is derived was not available to be compared with that of the tumors that occurred when NF1-90.8 was injected into athymic mice.

[d]Because the cells did not continue to grow in culture, cells were not available for additional experiments.

Figure 3. Hematoxylin and eosin stained sections of neurofibrosarcomas.

(*A*) Neurofibrosarcoma NF1-WSU-10 that occurred in patient with Type 1 neurofibromatosis. (*B*) Neurofibrosarcoma that occurred in athymic mouse when NF1-WSU-10 cells were injected subcutaneously into an athymic mouse. (*C*) Neurofibrosarcoma NF1-WSU-17 that occurred in patient with Type 1 Neurofibromatosis. (*D*) Neurofibrosarcoma that occurred in athymic mouse when NF1-WSU-17 cells were injected subcutaneously into an athymic mouse. (*E*) Neurofibrosarcoma that occurred in athymic mouse when NF1-90.8 cells were injected subcutaneously into an athymic mouse.


A
B
C
D
E

a second NF1 patient, respectively. These three cell lines were also tested for the ability to make tumors in athymic mice. NF1-90.8 produced progressively growing tumors with histological features consistent with that of human neurofibrosarcomas (see Figure 2).

RT-PCR assay to detect PDGF-B and TGF-α mRNA

Human neurofibromas have been shown to produce mitogenic growth factors including bFGF (11). However, bFGF and several other growth factors are not mitogenic for rat Schwann cells or Schwann cells derived from human neurofibromas unless cAMP is artificially elevated. Since neurofibrosarcomas have elevated GTP-ras, and in several cell types ras activation stimulates adenylate cyclase which subsequently elevates the level of cAMP, we reasoned that growth factors such as bFGF, PDGF-B, and other growth factors could be mitogens in neurofibrosarcoma cells. We have also previously observed in this laboratory that PDGF-B and TGF-α are both expressed in human fibrosarcomas (unpublished studies). Because of these observations, we investigated whether neurofibrosarcoma cells express PDGF-B and TGF-α at elevated levels.

Normal human cells used as controls included two normal human foreskin fibroblast lines, an adult smooth muscle cell line, and a fibroblast line that was derived from normal skin of the NF1 patient that developed the neurofibrosarcoma that gave rise to cell line NF1-90-8 (Table II). We also included cell lines derived from human malignancies

[a]Provided by Dr. Peter Libby, Brigham and Women's Hospital, Boston, MA

[b]Provided by Dr. Thomas Glover, University of Michigan, Ann Arbor, MI

[c]Tumors provided by Dr. Mark Zalupski, Wayne State Univ., Detroit, MI

[d]Tumors provided by Dr. William Mercer, Michigan Capital Medical Center, Lansing, MI

[e]Tumor provided by Dr. Mark Lebwohl, dermatologic surgeon, New York, NY.

[f]Cell lines from The American Type Culture Collection, Rockville, MD.

[g]Justin McCormick, unpublished studies

Table II. Human cell lines/strains

Cell line or strain	Cell type - origin
SL-80, SL-68	Human foreskin fibroblast cell lines derived from normal infants
HSVSMC.13	Human smooth muscle cells derived from a normal adult[a]
90.8 NF	Phenotypically normal fibroblasts derived from biopsy in NF1 patient that developed neurofibrosarcoma NF1 90.8[b]
NF1-WSU-4, NF1-WSU-10, NF1-WSU-17	Human Neurofibrosarcoma cell lines established at Michigan State University[c]
NF1 90.8, NF1 88.3, NF1 88.14	Cell lines derived from neurofibrosarcomas that occurred in patients with NF1[b]
SC #1-11	Neurofibroma cell lines derived from multiple neurofibromas that occurred on an NF1 patient[d]
NY-1	Neurofibroma cell line derived from neurofibroma that occurred on an NF1 patient[e]
HT-1080, VIP-FT	Human fibrosarcoma-derived cell lines[f]
MFH-WSU-1	Human malignant fibrous histiocytoma cell line established at Michigan State University[c]
MSU-1.1-v-*sis*	Malignant cell strain generated *in vitro* from immortal human fibroblast line, MSU-1.1 (Morgan et al) by transfection of a v-*sis* construct[g]
MSU-1.1-H-*ras*	Malignant cell strain generated *in vitro* by transfection of H-*ras* construct into MSU-1.1[g]

that arose from mesenchymal cells including HT-1080, VIP-FT, and MFH-WSU-1. Other cells used as controls were two malignant strains generated from the same immortal human fibroblast strain MSU-1.1. MSU-1.1 v-*sis* was produced by transfection with a v-*sis* oncogene and, as such, expresses high levels of PDGF-B mRNA. MSU-1.1 v-*ras* was generated by transfection with a v-H-ras oncogene and does not express either TGF-α or PDGF-B mRNA.

The ideal control cells to assess changes that have occurred to cause a neurofibroma would be the normal human Schwann cells. Unfortunately, such cells are not readily available and do not grow in standard cell culture. In any case, we doubt that normal Schwann cells express significant levels of growth factors, since Ratner et al. (11) demonstrated that extracts prepared from normal human peripheral nerves did not contain any mitogens, whereas neurofibroma extracts did. The most appropriate control cells to assess changes that cause neurofibrosarcomas are the neoplastic Schwann cells in neurofibroma cell populations.

RT-PCR

To determine if the neurofibrosarcoma and neurofibroma cell lines expressed TGF-α and PDGF-B mRNA, and if so, the relative level of expression, we used an RT-PCR assay. The primers that we used are shown in Figure 3. One out of the six neurofibrosarcoma cell lines tested expressed high levels of PDGF-B mRNA, and TGF-α mRNA equivalent to that of human fibrosarcoma cell line HT-1080 (Figures 4 and 5), which has previously been shown to express elevated levels of PDGF-B mRNA and protein (25) as well as TGF-α mRNA (26). Early passage neurofibromas cells were found to express intermediate or low levels of PDGF-B mRNA

Figure 3: PCR PRIMERS

GAPDH Primers:

5' sense primer
5' -GGTGAAGGTCGGAGTCAACG- 3'
(exon 2)

3' antisense primer
5' -CTTCTGCATGGTGGTGAAGA- 3'
(exon 5)

cDNA product = 313 bp

PDGF-B Primers:

5' sense primer
5' -GAAGGAGCCTGGGTTCCCTG- 3'
(exon 3 - exon 4 junction)

3' antisense primer
5' -TTTCTCACCTGGACAGGTCG- 3'
(exon 4 - exon 5 junction)

cDNA product = 226 bp

TGF-α:

5' sense primer
5' -GTATTGTGTTGGCTGCGTGC- 3'
(exon 2)

3' antisense primer
5' -CACTGTTTCTGAGTGGCACG- 3'
(exon 3)

cDNA product = 437 bp

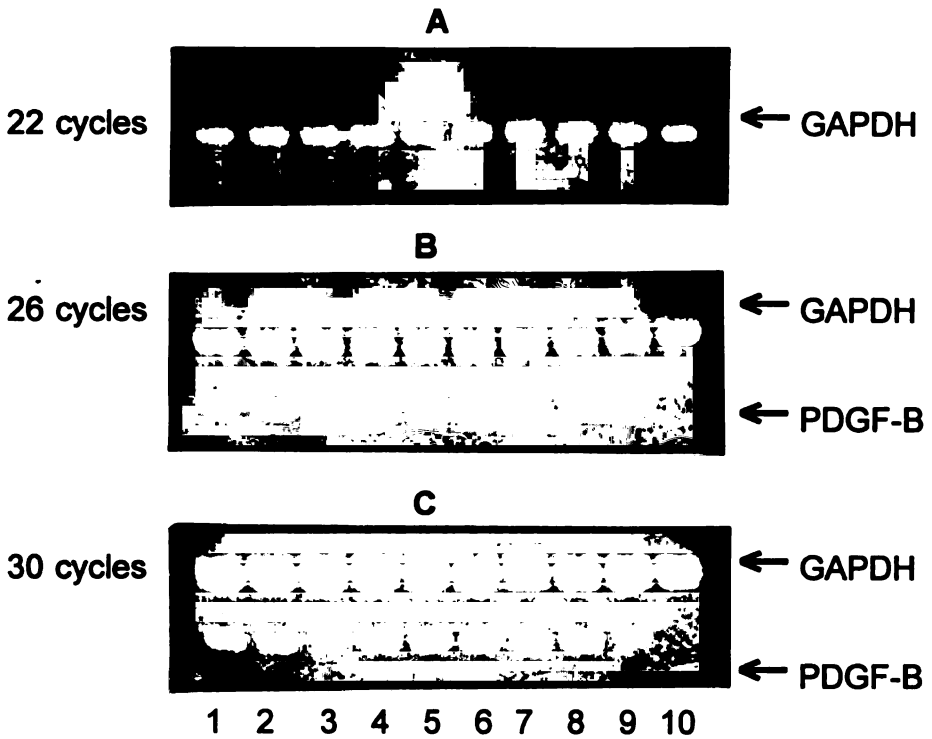


Fig. 4. Expression of GAPDH mRNA and PDGF-B mRNA determined by RT-PCR.

(A) Sampling of PCR reaction after 22 cycles showing GAPDH product. (B) Sampling of PCR product after 26 cycles also showing PDGF-B PCR product. (C) Sampling after 30 cycles of PCR. Lane 1, HT-1080; Lane 2, MFH-WSU-1; Lane 3, NF1-WSU-4; Lane 4, NF1-WSU-10; Lane 5, NF1-WSU-17; Lane 6, NF1-88.3; Lane 7, NF1-88.14; Lane 8, NF1-90.8; Lane 9, 90.8 NF; Lane 10, SL-80.

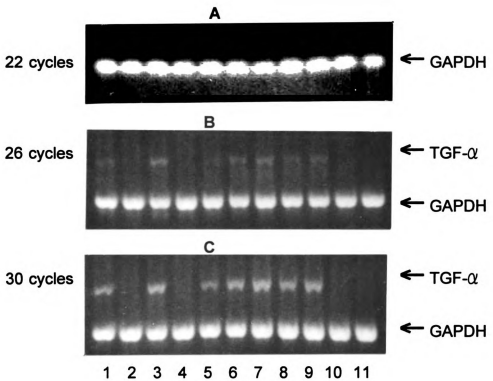


Fig. 5. Expression of GAPDH mRNA and TGF-α mRNA determined by RT-PCR.

(A) Sampling of PCR reaction after 22 cycles showing GAPDH product. (B) Sampling of PCR product after 26 cycles also showing TGF-α PCR product. (C) Sampling after 30 cycles of PCR. Lane 1, HT-1080; Lane 2, MFH-WSU-1; Lane 3, VIP-FT; Lane 4, NF1-WSU-4; Lane 5, NF1-WSU-10; Lane 6, NF1-WSU-17; Lane 7, NF1-88.3; Lane 8, NF1-88.14; Lane 9, NF1-90.8; Lane 10, 90.8 NF; Lane 11, SL-80.

(Figure 6). Only one neurofibroma cell line, NFb-1, was found to express both TGF-α mRNA and PDGF-B mRNA (data not shown). To exclude the possibility that neurofibroma cell line NFb-1, which expresses elevated TGF-α mRNA, became cross-contaminated by a TGF-α expressing tumor cell line during cell culture, we reestablished this neurofibroma cell line from tissue and cells that had been frozen at the time that tumor tissue was received from the hospital. Our findings confirmed that NFb-1 expresses elevated TGF-α mRNA.

As shown, normal human foreskin fibroblasts and human adult smooth muscle cells express low or undetectable levels of PDGF-B, and none of the normal cells expressed detectable TGF-α mRNA. All experiments were carried out at least twice and consistent results were obtained. The data from RT-PCR are representative of the data we obtained in several experiments. The results are summarized in Table III.

Response of neurofibrosarcoma cells to exogenous growth factors

In order to determine whether production of PDGF-B and/or TGF-α could have a direct effect on the growth of neurofibrosarcoma cells, we have used an assay in which cells are plated in medium lacking exogenous growth factors and then are stimulated over a period of 6 days, and at the end of the assay cell numbers in each culture dish are determined. If no growth was detected above that which normally occurs in growth factor free medium, this indicated either that the cells do not have functional receptors for that growth factor or that the growth of the cells is completely independent of exogenous growth factors. If significant growth occurred in response to a particular growth factor, it indicated that the cell

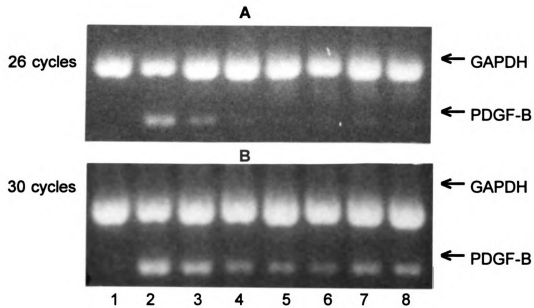


Fig. 6. Expression of GAPDH mRNA and PDGF-B mRNA determined by RT-PCR.

(A) Sampling of PCR reaction after 26 cycles showing GAPDH product and PDGF-B products. (B) PCR product after 30 cycles of PCR amplification. Lane 1, SL-80; Lane 2, NF1-WSU-17; Lane 3, NFb-1; Lane 4, NFb-2; Lane 5, NFb-5; Lane 6, NFb-7; Lane 7, NFb-9; Lane 8, NFb-10.

(data is based on several experiments)

[a]- = no PCR product detected after 30 cycles of amplification in any experiment

[b]+ = light band of PCR product visualized at 30 cycles in some experiments

[c]++ = a light band was visualized at 26 cycles in some experiments

[d]+++ = bright band of PCR product visualized at 26 cycles of amplification

[e]++++ = PCR product visualized at 22 cycles in some experiments

Table III. Results of RT-PCR

Cells	TGF-α	PDGF-B
<u>Normal cells</u>		
SL80	-[a]	-
SL68	-	+[b]
HSVSMC.13	-	-
90.8	-	+
<u>Sarcoma cells</u>		
HT 1080	++[c]	+++[d]
VIP-FT	+++	+++
MSU-1.1-v-*sis*	-	++++[e]
MSU-1.1-H-*ras*	-	-
<u>Neurofibrosarcoma cells</u>		
NF1-WSU-4	-	+
NF1-WSU-10	++	+++
Nf1-WSU-17	++	+++
NF1 88.3	++	+++
NF1 88.14	++	++
NF1 90.8	++	+++
<u>Neurofibroma cells</u>		
NFb-1	++	++
NFb-2	-	++
NFb-3	-	+
NFb-4	-	+
NFb-5	-	+
NFb-7	-	+
NFb-9	-	++
NFb-10	-	++
NFb-12	-	+

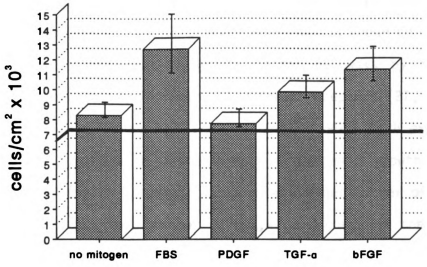
lines expressed functional receptors for that growth factor. We used the three tumorigenic human neurofibrosarcoma cell lines that grow continuously in standard culture medium containing serum, and grow to some degree in medium lacking exogenous growth factors. NF1-WSU-10 proliferates rapidly in the absence of growth factors, whereas NF1-90.8 grows moderately and NF1-WSU-17 grows slowly in these conditions. We also included bFGF in addition to PDGF and TGF-α in the assay because of reports that bFGF is found in extracts obtained from human neurofibromas (11) and is mitogenic for rat Schwann cells in the presence of elevated cAMP (16). The results demonstrate that neurofibrosarcoma cell line NF1-90.8 responds markedly to TGF-α, PDGF, and bFGF, indicating that receptors for these growth factors are expressed (Figure 7). NF1-WSU-17 responds to TGF-α and bFGF, but is not stimulated by PDGF. None of the growth factors that we used had a stimulatory effect on neurofibrosarcoma cell line NF1-WSU-10 (data not shown). These results indicate that neurofibrosarcoma cell lines NF1-90.8 and NF1-WSU-17, in contrast to normal rat Schwann cells and human neurofibroma-derived Schwann cells, constitutively express one or more of the EGF/TGF-α, PDGF, and bFGF receptors.

Effect of suramin on growth factor independence

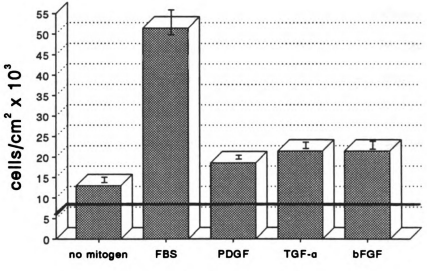
If growth factors such as PDGF-B and bFGF are produced by neurofibrosarcomas and have a direct role in the abnormal growth of such cells, then inhibiting the interaction between growth factors and their receptors in such cells should have an inhibitory effect on cell growth. Because two of the neurofibrosarcoma cell lines, NF1-WSU-10 and NF1-90.8, continue to divide in medium lacking growth factors, we could examine

Figure 7. Stimulation of the growth of neurofibrosarcoma cells by exogenous growth factors. The cells were grown in McM with 0.1 mM calcium and SR_2 (no mitogen), or in McM with 0.1 mM calcium and SR_2 plus 10% FBS (FBS), or in McM with 0.1 mM calcium and SR_2 plus 10 ng/ml PDGF (PDGF), or McM with 0.1 mM calcium and SR_2 plus 10 ng/ml TGF-α (TGF-α), or McM with 0.1 mM calium and SR_2 plus 10 ng/ml bFGF (bFGF). The number of cells at the end of the assay are shown in cell/cm_2. The bold horizontal line demonstrates the number of cells present at the time cells were changed to experimental conditions.

NF1-WSU-17



NF1-90.8



whether inhibiting growth factor initiated mitogenesis would have a negative effect on the serum free growth of these cells. The other neurofibrosarcoma cell lines and all neurofibroma cell lines do not divide, or divide very slowly (NF1-WSU-17), under these conditions (data not shown). Suramin is an antifilarial drug that is known to inhibit cell division that is dependent on growth factor pathways by interrupting interactions between growth factors and their receptors (27, 28). Since neurofibrosarcoma cell lines NF1-WSU-10 and NF1-90.8 both express TGF-α and PDGF-B mRNA, and divide in the absence of exogenous growth factors, we treated the cells with suramin to determine whether either of these cell lines depends upon growth factor pathways for growth in the absence of exogenous growth factors. As control cells we used two tumorigenic cell lines derived from the same parental human fibroblast cell line, one generated by transfection with an activated H-*ras* gene (MSU-1.1-H-*ras*) and the other generated by transfection with a v-*sis* gene (MSU-1.1-v-*sis*) (29). Both of these malignantly transformed cell lines grow rapidly in serum free medium. However, as shown in Figure 8, MSU-1.1-v-*sis*, is completely inhibited from growing in serum free medium in the presence of 50μg/ml suramin. The serum free growth of cell strain MSU-1.1-H-ras, is unaffected by 50μg/ml suramin. Like MSU-1.1-H-*ras*, neurofibrosarcoma cell line NF1-WSU-10 is unaffected by 50μg/ml of suramin. However, the growth of neurofibrosarcoma line NF1-90.8 in growth factor free medium is completely inhibited by 50 μg/ml of suramin (Figure 8). These findings suggest that neurofibrosarcoma-derived cell line NF1-90.8 is dependent upon endogenous production of a growth factor(s) to proliferate in the absence of exogenous growth factors.

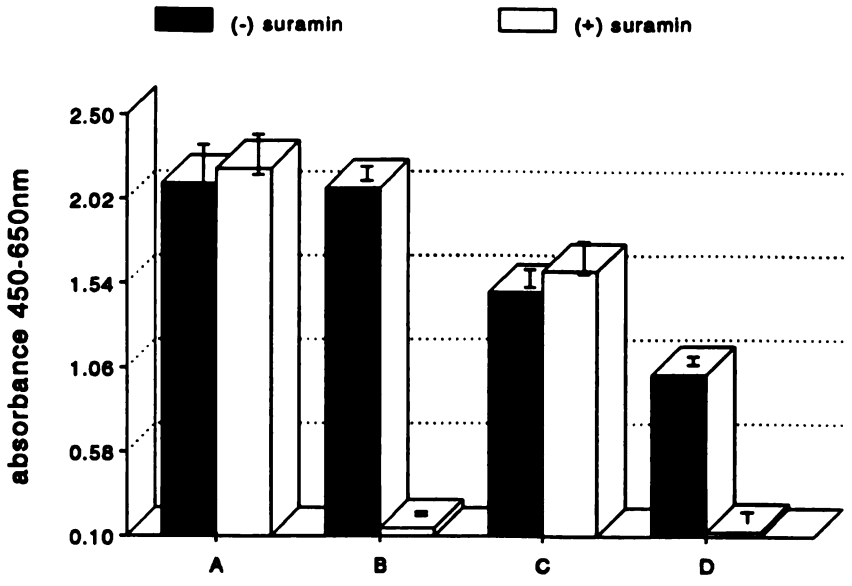


Figure 8. Growth of tumor cell lines in McM medium with 0.1 mM calcium and SR_2 (black bar), and in McM medium with 0.1 mM calcium and SR_2 plus 50 μg/ml of suramin (white bar). (*A*), MSU-1.1-H-*ras*. (*B*), MSU-1.1-v-*sis*. (*C*), NF1-WSU-10. (*D*), NF1-90.8. Growth is shown as a function of absorbance calculated by subtracting the absorbance determined at day zero from the absorbance determined at the end of the assay using the colorimetric assay described in materials and methods.

Discussion

In the present study, we have shown evidence that supports a role for activation of growth factor pathways, i.e., PDGF-B and TGF-α, in the pathogenesis of neurofibrosarcomas that occurs in NF1 patients. Our results clearly demonstrate that the expression of TGF-α mRNA is associated with malignant transformation of neurofibroma into neurofibrosarcomas (Table III), since with only one exception, neurofibroma cell lines do not express TGF-α mRNA but neurofibrosarcoma cells do. One possible explanation for this result is that neurofibroma cell line NFb-1 is actually derived from a tumor that is at an intermediate stage between a neurofibroma and a neurofibrosarcoma.

It is clear that the majority of neurofibrosarcomas have elevated expression of PDGF-B mRNA relative to neurofibroma cell lines. However, we cannot exclude to possibility that the neoplastic Schwann cells in neurofibroma cell populations also express levels of PDGF-B mRNA similar to that of neurofibrosaroma cells, but that the concentration of PDGF-B mRNA has become diluted due to the presence of the RNA from non-neoplastic cells that are present in neurofibroma cell populations. In addition, since we could not compare the level of PDGF-B mRNA to that of normal human Schwann cells, it is not possible to determine whether the level of PDGF-B mRNA expression by neurofibroma cells represents upregulation or a steady-state level of expression in normal human Schwann cells. Nevertheless, it seems unlikely that normal Schwann cells produce PDGF-B, since other workers have demonstrated that soluble extracts from normal human nerve do not contain any factors that are mitogenic for NR6-3T3 fibroblasts (11).

Further evidence that growth factor expression can play a critical role in the abnormal growth of neurofibrosarcomas is based on studies with

suramin. Suramin has been used to inhibit the growth of malignant cells generated in cell culture by growth factor oncogenes (27, 28). There are few examples in which inhibition of a growth factor pathway has been shown to have an inhibitory effect on growth of human tumor-derived cells (30, 31). Our results demonstrate that NF1-90.8 cells, just like those malignantly transformed by v-*sis*, are inhibited by 50 μg/ml of suramin. These results demonstrate that NF1-90.8 cells depend upon endogenous production of a mitogenic growth factor(s) to proliferate in the absence of exogenous growth factors.

Lemke (32) has proposed that the abnormal phenotype of neoplastic Schwann cells in NF1 patients could in part be due to abnormal regulation of ras and subsequent upregulation of growth factor receptor(s). Defective regulation of ras proteins in yeast leads to elevation of cAMP (33). Activation of adenylate cyclase and subsequent increase in cAMP can have a causal role in some types of human tumors. For example, a group of human pituitary tumors have been identified that have constitutively elevated levels of cAMP (3). The mutations that cause elevation in cAMP are in the α_s subunit of G_s, a ras-related protein that functions in activation of adenylate cyclase in pituitary and other types of cells. Mutations in the α_s subunit, which is the GTP-binding domain of G_s proteins, results in decreased GTPase activity and therefore to elevation of GTP bound to G_s, which is the active form. Consequently, adenylate cyclase is constitutively activated and continuously generates cAMP in these pituitary tumors. If a similar mechanism occurs in human Schwann cells from NF1 individuals who that express reduced amounts of neurofibromin, as suggested by Lemke, then the constitutional NF1 defect alone does not appear to be sufficient to result in constitutively activated adenylate cyclase, since neurofibroma Schwann cells do not

respond to growth factors in the absence of forskolin (11, 12). On the other hand, the human neurofibrosarcoma cell lines studied by DeClue et al. (10) have elevated ras-GTP. If adenylate cyclase is activated in neurofibrosarcoma cells, as a result of their constitutively activated ras proteins, then it is likely that such cells will have upregulated growth factor receptor expression as a result of adenylate cyclase-induced elevation of cAMP.

In vitro studies suggest that rat Schwann cells are similar to human Schwann cells in their regard to their expression of growth factor receptors, since Schwann cells derived from human neurofibromas also require elevated cAMP to respond to specific growth factors. Glial growth factor (GGF) was the first Schwann cell mitogen identified and stimulates rat Schwann cells in culture in the absence of elevated cAMP (16). Ridley et al (14) demonstrated that TGF-β1 and TGF-β2, like GGF, also stimulate rat Schwann cells to proliferate in the absence of elevated cAMP. Davis and Stroobant (16) screened several mitogens to determine which, if any, could stimulate rat Schwann cells in culture in the absence or presence of cAMP. Their results verified that GGF, TGF-β1 and TGF-β2 stimulate growth of rat Schwann cells in the absence of cAMP, and they found that PDGF-BB, PDGF-AB, bFGF, and aFGF could also stimulate the growth of rat Schwann cells, but only in the presence of forskolin-induced elevation of cAMP. cAMP induces Schwann cells to become sensitive to the mitogenic effects of PDGF-B by elevating the expression of PDGF receptors (15). In the light of these facts, our results imply that two significant changes have occurred to neurofibrosarcomas compared to the neoplastic Schwann cells in neurofibromas. First, we predict that neurofibrosarcoma cells do not require exogenous stimulation of cAMP to express growth factor receptors, suggesting that such cells have elevated levels of cAMP. Second, since

neurofibrosarcomas can (to various degrees) replicate in the absence of serum or growth factors, our results suggest either that neurofibrosarcomas produce endogenous growth factors or that they have acquired other changes which preclude the need for growth factors in cell division.

Expression of a growth factor(s) by neurofibrosarcoma cells can be expected to would likely have two consequences in regard to signal transduction. First, growth factor expression would further elevate the level of ras-GTP by activation of guanine nucleotide releasing factors. It is possible that the effect of growth factor-induced activation of ras would be magnified in neurofibrosarcoma cells, since these cells lack neurofibromin (10) required to inactivate ras-GTP. Second, by elevating the level of ras-GTP and subsequently that of cAMP, production of a specific growth factor, such as PDGF-B, would further elevate the level of expression of its own receptor and possibly other growth factor receptors.

To summarize, our findings provide evidence consistent with the findings of others suggesting a role for mitogenic growth factors in the pathogenesis of neurofibromas and neurofibrosarcomas that occur in NF1 patients. Furthermore, our results add to the understanding of how Schwann cells in individuals affected with NF1 can become malignant. Previously, other workers have shown that loss of the remaining normal NF1 allele is associated with malignant transformation of neurofibromas (10). This finding is consistent with that of Menon et al (34), who demonstrated that the majority of neurofibromas retain the normal NF1 allele. Loss of heterozygosity of the p53 allele has also been associated with the formation of neurofibrosarcomas (34). Our findings suggest that expression of growth factors, including PDGF-B and TGF-α, and the ability to respond to such growth factors, is a characteristic of some human

neurofibrosarcoma cells. It is clear that future progress in understanding the molecular details involved in the formation of neurofibromas and neurofibrosarcomas depends on understanding the mechanism whereby growth factor receptors become upregulated in human Schwann cells, and the connection, if any, between ras activation and activation of adenylate cyclase in such cells.

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CHAPTER III

Characterization of a Neoplastic Human Fibrous Histiocytoma Cell Line Including Evidence Suggesting a Mesenchymal Origin

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ABSTRACT

In this study we characterize a human malignant fibrous histiocytoma (MFH) cell line that was derived from a recurrent MFH that occurred in a 72 year old male. Several morphological types of cells were observed in the primary culture. After several population doublings a homogeneous infinite life span cell line, designated MFH-WSU-1, which consisted of ovoid-spindle shaped cells and frequent multi-nucleated giant cells, remained. When injected into athymic mice, the MFH-WSU-1 cells produced a tumor histologically identical to the original tumor indicating that the cells are tumor-derived. This cell line expressed platelet-derived growth factor B-chain (PDGF-B) mRNA at a level similar to that of human fibrosarcoma cell line HT-1080 and several other human fibrosarcomas. Furthermore, MFH-WSU-1 proliferates in response to exogenous transforming growth factor alpha (TGF-α). Since these growth factors and receptors are frequently associated with tumors of mesenchymal origin and not associated with cells of monocyte/macrophage origin, these results support a mesenchymal origin for this MFH cell line.

INTRODUCTION

Malignant fibrous histiocytoma (MFH) is the most common soft tissue malignancy (4,8). This diagnosis describes a histologically heterogeneous group of sarcomas. The majority of MFHs are of the storiform-pleomorphic pattern (4). Other less common forms include the myxoid, giant cell, inflammatory, and angiomatoid types. Despite the fact that MFH is the most commonly diagnosed soft tissue malignancy, there are very few reports describing and characterizing established human MFH cell lines.

The cell type from which MFHs arise has been the subject of much controversy. The designation of MFH was originally used because investigators observed that MFH explants give rise to cells in culture that resemble histiocytes (9). Later passage cells appeared fibroblastic and it became generally accepted that histiocytes were able to assume the function and appearance of fibroblasts. Conflicting results have been reported in investigations that used immunocytochemical methods to demonstrate the presence of mesenchymal or histiocytic markers in MFH-derived cells. Several reports conclude that MFH cells are of mesenchymal origin (11,12,7,18). Conversely, other reports indicate that MFHs are of histiocytic origin (16,14,15,1). Each of these studies relies on the specificity of cell markers. However, one of the characteristics of MFHs is that they are composed of a diverse cell types, including neoplastic and nonneoplastic cells. Therefore, data from studies of fixed tumor tissue or primary cell cultures must be interpreted with caution, since it

is often unclear which cells are neoplastic and which are not. In the present study we characterize a newly established MFH cell line and we provide data on the malignant cells that suggest that the tumorigenic cells are of mesenchymal origin.

MATERIALS AND METHODS

Culture of MFH cells

Tumor tissue was obtained from a fresh human tumor that had been surgically excised and was transported to our laboratory in Lebovitz medium containing 100 μg/ml Gentamicin sulfate. Upon arrival at this laboratory, the tissue was fragmented into smaller pieces and mechanically agitated using a sterile syringe and cannula to break down the tissue and free individual cells. Some of the resulting cells and small tumor fragments were placed in 75 cm^2 tissue culture flasks (Corning Glass Works, Corning N.Y.) and cultured in Eagle's minimum essential medium supplemented with 0.2 mM aspartic acid, 1.0 mM sodium pyruvate, 0.2 mM serine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml hydrocortisone, and 5% or 10% fetal bovine serum (FBS) (culture medium). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and air.

Cloning Assay

Cells were plated at 100, 200, or 500 cells per 100 mm-diameter culture dish in McM medium (13) containing 10% supplemented calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml hydrocortisone. Medium was changed weekly. Cell clones were stained after 3 weeks.

Saturation Density Assay

Cells were plated at 5 x 10^4 cells per 60 mm-diameter culture dishes in 5 ml of culture medium containing 5% FBS. Medium was changed every 3 days. Duplicate culture dishes for each cell line were counted on the indicated days after plating.

Tumorigenicity Assay

Cells (1 x 10^6 to 10 x 10^6) in exponential growth were trypsinized, suspended in a total volume of 0.2 ml of Eagle's medium and injected subcutaneously in the scapular region of six week old BALB/c athymic mice. The mice were examined weekly for tumors. Tumors that were removed were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin prior to histological evaluation.

Isolation of RNA

Cells were grown in 150 mm-diameter dishes in culture medium containing supplemented calf serum rather then fetal bovine serum. Total RNA was isolated from cells just prior to their reaching confluence, using the procedure of Chomczynski and Sacchi (2) essentially as described. The RNA was resuspended in diethylpyrocarbonate-treated H_2O, and stored at -80°C. The concentration of RNA was determined from the optical density at 260nm, and the integrity of the RNA was qualitatively determined from the appearance of ribosomal RNA bands on 1% agarose/formaldehyde ethidium bromide gels.

Detection of mRNA by RT-PCR

Synthesis of cDNA using Moloney murine leukemia virus reverse transcriptase (New England Biolabs) and amplification of specific cDNAs

using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), RT-PCR, was carried out using the procedure described by Yang et al (19). The primers used for glyceraldehyde phosphate dehydrogenase (GAPDH) and TGF-α cDNA have been described by Vassar et al. (17) and primers for PDGF-B cDNA are described by Yang et al. (19).

Response of MFH cells to PDGF-B and TGF-α

To determine the response of cells to specific growth factors, cells growing exponentially in culture medium were trypsinized and plated at 7.5 x 10^4 cells per 60 mm-diameter culture dish in 5 ml of culture medium with 5% fetal bovine serum. After 24 h, the medium was removed and replaced with 5 ml of medium containing McM medium modified to contain only 0.1 mM calcium, rather than the usual 1.0 mM calcium, and containing the serum replacement supplements (designated SR_2) specified by Ryan et al. (13), but lacking any exogenous protein growth factors plus 1% FBS. Fetal bovine serum was added to positive growth control conditions to achieve a concentration of 10% FBS. Growth factors including PDGF-B (Oncogene Science, Uniondale, N.Y.) and TGF-α (Oncogene Science) were added to achieve a final concentration of 2 ng/ml or 10 ng/ml. Cells in triplicate 60 mm-diameter culture dishes were counted on day 8.

RESULTS

MFH Cells in Culture

Cells and tissue from the MFH gave rise to a primary cell culture that consisted of a heterogeneous population of cells (Fig. 1). We reduced the concentration of serum present in culture medium to 5% and found that this allowed a more homogeneous population of cells to overgrow

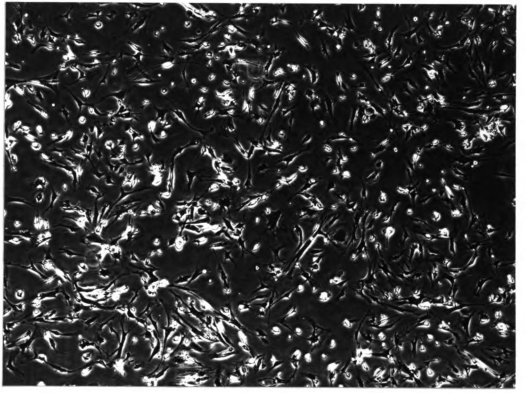


Figure 1. Early passage (passage two) MFH-WSU-1 cells in culture demonstrating variety of morphological types of cells in primary culture.

the rest the cells in primary cultures. These cells appeared morphologically transformed and had an ovoid-spindle shaped appearance in cell culture (Fig. 2). These cells have continued to divide in cell culture for more than 200 population doublings. To verify that the malignant cells were present in the primary population of cells we thawed cells and tissue that had been frozen at the time the human tumor was received and placed the material in culture medium that contained only 1% supplemented calf serum. The predominant cell type that grew out under these conditions had cell morphology identical to the infinite life span cells that eventually grew out of the cultures in standard culture medium. These results suggest that the malignant cells are present in the primary culture.

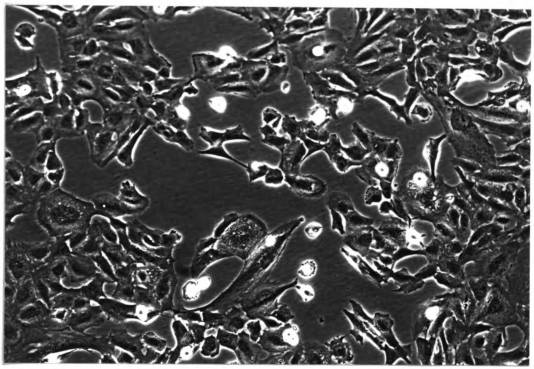
Several clonal populations were isolated from the heterogeneous primary cultures, and passaged as individual cell strains. These strains demonstrated a variety of morphologies, including fibroblastic and histiocytic strains. However, each of the cell strains senesced in culture when passaged continuously. Cloning assays of the homogenous late passage cell line have demonstrated that the cloning efficiency of these cells is less than one percent, and explains why we did not isolate these cells from cloning assays of the early passage culture primary cells.

Saturation Density of MFH-WSU-1

We used a saturation density assay to compare the growth of this MFH cell line, designated MFH-WSU-1, to that of normal human fibroblasts and human fibrosarcoma-derived cell line HT-1080. Under the conditions used, the MFH-WSU-1 cells proliferated considerably more slowly than HT-1080, but over time reached a density only 2-fold lower than that of HT-1080 (Fig. 3). MFH-WSU-1 grew at a rate similar to that of the normal

Figure 2. Morphology of infinite life span cell line derived from human MFH. (A) MFH cells in culture including ovoid to fibroblastic cells and several multi-nucleated giant cells (arrows). (B) Higher power of MFH cells in culture including a multi-nucleated giant cell (arrow).

A



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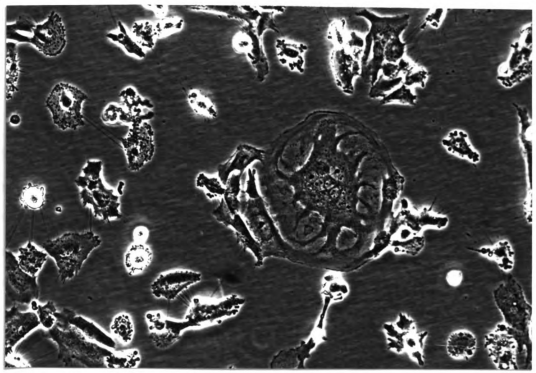


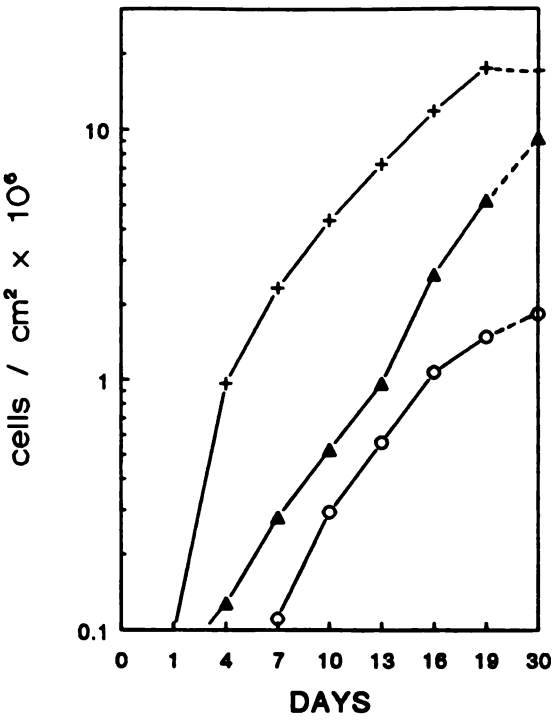


Figure 3. Saturation density assay. Cells were plated and cultured as described. (+) represents growth of human fibrosarcoma cell line HT-1080. (o) represents normal human foreskin fibroblasts. (◂) represents human MFH cell line MFH-WSU-1. Cell growth is calculated as the number of cells per cm^2.

fibroblasts until the cells became newly confluent. Under these conditions, the MFH-WSU-1 cells were able to continue dividing at the same rate, whereas the rate of growth of the normal fibroblasts slowed as the density increased. As a result, MFH-WSU-1 had achieved a 5-fold greater density than the normal fibroblasts by the time the assay was ended on day 30.

Tumorigenicity Assay

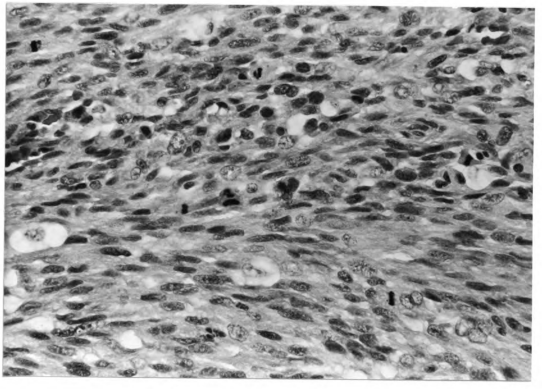
Both early or late passage MFH-WSU-1 cells that were injected subcutaneously into athymic mice produced tumors. However, if such tumors were not removed within approximately two months they eventually regressed. A total of 8 mice were injected with these cells. Tumors formed in seven of the eight mice and the tumors grew to approximately 0.5 cm in diameter. The two tumors that were removed had a histological pattern similar to the original human tumor (Fig. 4). Microscopic examination of hematoxylin and eosin stained sections demonstrated numerous mitotic figures in the tumor mass. We also noted the presence of numerous mononuclear cells at the periphery of the tumor mass that appeared to be invading tumor tissue (Fig. 5), suggesting that the regression of these tumors occurred as the result of residual immunological response of the athymic mice.

Growth Factor mRNA Expression

Investigators have previously reported that human fibrosarcoma-derived cell lines, such as HT-1080, express elevated levels (relative to normal human fibroblasts) of mitogenic growth factors including TGF-α (3) and PDGF-B (10). McCormick and his colleagues have also found that human fibrosarcoma cell lines frequently express both of these growth factors

Figure 4. Hematoxylin and eosin stained tumor sections. (A) Original human tumor that occurred in a 72 year old male and gave rise to cell line MFH-WSU-1 in culture. (B) Tumor that occurred when MFH-WSU-1 cells were injected subcutaneously into an athymic mouse, as described.

A



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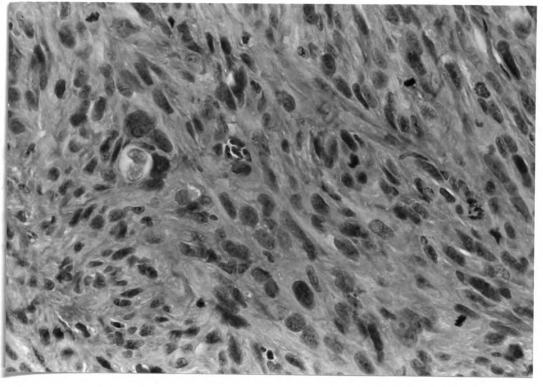

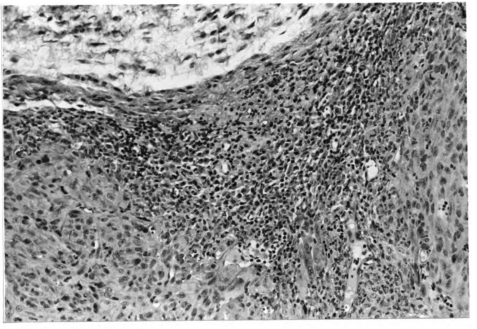

Figure 5. Hematoxylin and eosin stained section of tumor produced by MFH-WSU-1 when cells from culture were injected subcutaneously into an athymic mouse as described. This field demonstrates infiltration of host mononuclear cells into the neoplastic tissue.

(unpublished results). The results showed that MFH-WSU-1 expresses levels of PDGF-B mRNA similar to or higher than that of HT-1080, but WSU-1 do not express TGF-α mRNA (Fig. 6).

Response of MFH-WSU-1 to mitogenic growth factors

MFH-WSU-1 divide slowly in the absence of exogenous growth factors or serum. MFH-WSU-1 cells responded markedly to 2 ng/ml or 10 ng/ml TGF-α in the absence of any other growth factor, indicating that this MFH cell line expresses functional receptors for this growth factor (Fig. 7). However, MFH-WSU-1 did not respond to recombinant PDGF-B, suggesting that this cell line does not express the appropriate receptor for this growth factor.

DISCUSSION

Studies of this MFH cell line have demonstrated that several different morphological cell types were present in primary cultures prepared from the original tumor. These results are similar to those in the early studies of other investigators who identified several cell types including those with histiocytic features (9). None of the clonally-derived independent cell strains isolated from primary MFH-WSU-1 cell populations, and chosen because they represented the various predominant cell types making up that population, proved to have an infinite life-span. The cells that eventually grew out of the tumor-derived tissue and continued to divide indefinitely did not resemble any of the cell strains isolated in various cloning experiments. These findings suggest that several types of cells present in primary cultures do not represent the neoplastic cells that formed the human tumor. The infinite life span cell

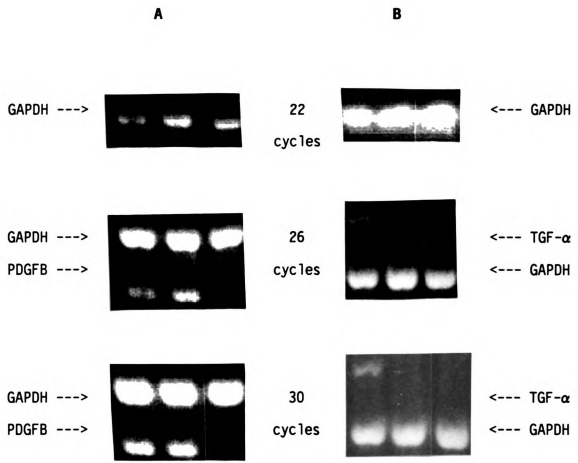

Figure 6. Expression of GAPDH, PDGF-B, and TGF-α mRNA determined by RT-PCR. PDGF-B and GAPDH mRNA expression shown in panel A. TGF-α and GAPDH mRNA expression shown in panel B. Lane (1), HT-1080. Lane (2), MFH-WSU-1. Lane (3), Normal human foreskin fibroblasts.

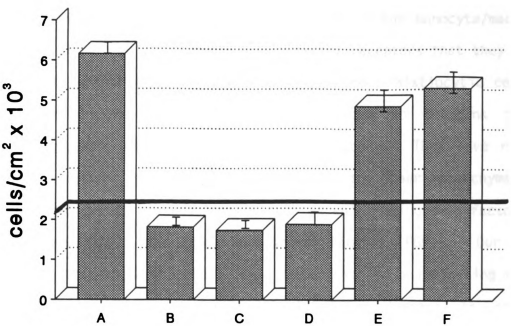

Figure 7. Growth response of MFH-WSU-1 cells to PDGF-BB and TGF-α. Growth is shown as cells per cm^2. The bold horizontal line represents the number of cells present at the time that the cells were changed to the experimental conditions as described. All conditions include McM medium containing 0.1 mM calcium and SR_2 plus the following as listed: (A) 10% FBS, (B) no growth factor added, (C) 2 ng/ml PDGF-BB, (D) 10 ng/ml PDGF-BB, (E) 2 ng/ml TGF-α, (F) 10 ng/ml TGF-α.

line that eventually grew out of the primary cell culture population formed tumors in athymic mice, and these tumors had similar histological features similar to that of the original human tumor, indicating that these cells are the *bone fide* MFH cells.

Cells of mesenchymal origin and those of the monocyte/macrophage lineage differ in the specific growth factor receptors that they express and those growth factors that they respond to. Histiocytic cells and other cells of hematopoietic origin respond to mitogens such as granulocyte/macrophage-colony stimulating factor. They have not been shown to respond to TGF-α (6). Fibroblasts and other mesenchymal cells have receptors for growth factors such as epidermal growth factor (EGF) and TGF-α, PDGFs, and fibroblast growth factors (FGFs). Our results indicate that these MFH-WSU-1 cells respond to TGF-α indicating that the receptor for this growth factor, the EGF/TGF-α receptor, is present on these cells. These receptors have been identified on many types of solid tumors, including those of mesenchymal origin, but have not been associated with cells of the monocytic/macrophage lineage or tumors that arise from these cells. We have also found that MFH-WSU-1 cells express elevated levels of PDGF-B mRNA, which is a characteristic of human fibrosarcoma-derived cell line HT-1080 and several other human fibrosarcoma-derived cell lines. However, we found that MFH-WSU-1 cells did not respond to PDGF-B, a potent mitogen for normal human fibroblasts. One possible explanation for this observation is that MFH-WSU-1 cannot respond to exogenous PDGF-B because the level of PDGF-B produced endogenously is already at a saturating level. Alternatively, MFH-WSU-1 may not have receptors for PDGF-B and the elevated production of PDGF-B, which is suggested by RT-PCR results, could exert some role in stromal formation or neovascularization (5).

The tumors that occurred when MFH-WSU-1 cells were injected regressed if they were not removed. When static or regressing tumors were removed and evaluated microscopically, several mitotic figures were observed in each high power field, indicating that despite the shrinking size of the tumor mass, the malignant cells continued to divide. This observation suggests that residual immunological factors in athymic mice can effectively destroy MFH-WSU-1 cells (20). We also observed mononuclear cells invading the tumors that occurred in athymic mice (Fig. 5), suggesting that MFH-WSU-1 cells secrete chemotactic factors and that such mononuclear cells contribute to destruction of the tumor cells. The infiltration of immune cells into the tumor mass is a known characteristic of MFH (20).

The overall slow rate of growth of MFH-WSU-1 cells *in vivo* relative to other human-derived malignant cell lines, such as HT-1080 (Fig. 3), could also contribute to the ability of mouse factors to cause regression of these tumors. However, since in this laboratory we have observed that other human tumor-derived cell lines, not of MFH origin, also grow slowly in culture and in athymic mice, but produce progressively growing tumors capable of killing the mice, slow growth alone is not enough to account for regression of the tumors produced by MFH-WSU-1 cells in athymic mice.

In conclusion, we have established and characterized a cell line derived from a human MFH, identified the malignant cells and demonstrated these cells produce tumors in athymic mice that are consistent with the diagnosis of a MFH. The numerous studies that have relied on immunohistochemical markers in fixed human MFH tissue to determine the cell of origin have two weaknesses. First, MFH tissue contains several types of cells including many non-neoplastic cells, and as a result studies of fixed tissue are difficult to interpret. Second, the majority

of the immunocytochemical markers routinely do not distinguish between mesenchymal and histiocytic cells. We have demonstrated that MFH-WSU-1 cells produce PDGF-B mRNA and respond to exogenous TGF-α. The receptors for these and other growth factors are commonly associated with cells that give rise solid tumors including sarcomas and carcinomas (6) but are not usually associated with tumors that arise from hematopoietic cells. It is possible that these or other receptors may be useful markers in distinguishing between tumors originating from mesenchymal cells and those of histiocytic origin.

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CHAPTER IV

Fibroblasts Derived from Normal Skin Biopsies from a Child with Infantile Myofibromatosis have Abnormal Growth Characteristics

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ABSTRACT

Infantile myofibromatosis is a rare disease in which neonates are born with benign lesions composed of fibroblastic or myofibroblastic cells. In most cases, the prognosis is good with eventual spontaneous regress of the tumors. In some instances, the lesions may involve the viscera and possibly cause death. This report describes a patient who was diagnosed with infantile myofibromatosis shortly after birth. This case in unusual because the lesions have neither been life threatening nor have they regressed. The patient is currently eight years old. We obtained two biopsies of apparently normal skin at the site of a surgical procedure. This report describes the studies of fibroblasts derived from these biopsies and demonstrates that such cells have abnormal growth characteristics including the ability to form foci in medium containing 1% serum and the ability to form benign tumors when injected into athymic mice. Fibroblasts derived from the skin of normal humans do not exhibit similar characteristics.

INTRODUCTION

Infantile myofibromatosis (IM) is an uncommon congenital syndrome that is characterized by the presence of benign tumors that occur in the skin, subcutaneous tissues, skeletal muscle, bone, and internal organs (2). The clinical course is variable. Typically, more than half of the tumors are present at birth, and the occurrence of new tumors is common only in the first two years of life. The majority of cases in which lesions are limited to soft tissues and bone generally carry a good prognosis including eventual spontaneous regression of the tumors. When there is considerable involvement of the viscera the prognosis is poor, and death often occurs at birth or shortly thereafter.

The etiology of IM is not known. The majority of cases of IM are sporadic. However, there are several reports of a familial pattern of inheritance (12,1), suggesting that the disease is either an autosomal dominantly inherited disease with reduced penetrance or an autosomal recessive inherited disease, with the former being the most favored (8). In either case, it appears that affected individuals carry an underlying genetic defect that predisposes them to the abnormal proliferation of fibroblasts and/or myofibroblasts.

This report includes a short clinical case report of an affected individual with an atypical clinical course. In addition, we describe studies of skin fibroblasts derived from two normal biopsies that were obtained at the time of an orthopedic procedure from an unaffected area of

skin of this patient. The fibroblasts from this patient exhibited abnormal growth in culture and produced benign nodules when injected into athymic mice.

CASE REPORT

This Caucasian male infant was delivered by caesarean section to a healthy mother after an uneventful term pregnancy. Apgar scores were 9 and 10. In the post delivery period hard lumps were noted on his back, legs and tongue. His right foot was flaccid and in severe dorsiflexion. Otherwise, the infant appeared healthy and in no distress.

A lesion was removed from the left lateral chest wall for histological diagnosis. Microscopic examination of hematoxylin and eosin stained sections revealed a well circumscribed nodule composed of a proliferation of spindle shaped cells in whorls, fascicles and bundles, which in many areas infiltrate through skeletal muscle. The spindle shaped cells were relatively uniform and elongate, but in some areas had distinctly plump, blunt ended nuclei and relatively abundant amphophilic cytoplasm. There were up to 2-3 mitoses per 10 high power fields with no atypical mitoses noted. Immunoperoxidase staining performed for S-100 protein was negative except for neural elements within the nodule. Electron microscopy examination of the proliferating fusiform cells demonstrated numerous profiles of slightly dilated reticulum as well as prominent Golgi zones. External lamina were sparse. The periphery of many of the cells contained myofilaments in association with Bence bodies. The nuclei were ovoid with dispersed chromatin and large active-appearing nucleoli. These findings demonstrate that the spindle shaped cells have features of fibroblasts and myofibroblasts.

This child, who is now 8 years old, continues to have new lesions arise. Magnetic resonance imaging has been used to follow the occurrence of these lesions. These have developed on the right breast and beside the trachea. The patient also has cutis marmorata (mottled atrophic appearing change) over extensive areas of the skin, especially on the right side of the face and neck, back, and legs. Biopsy of these regions revealed no specific findings. Other health-related problems include recurrent otitis media, tympanic membrane perforations, and recurrent sinusitis. The patient has undergone surgery for the deformities of his right foot.

MATERIALS AND METHODS

Culture of IM cells

Skin biopsies were obtained at the time that the patient underwent surgery for the deformity of his right foot. Two biopsies were taken from the margin of the surgical incision. The skin at this site appeared normal. The biopsies were fragmented and then agitated using a sterile syringe and canula to free individual cells. The resulting mixture of tissue fragments and individual cells was plated in culture medium that consisted of Eagle's minimum essential medium supplemented with 0.2 mM aspartic acid, 1.0 mM sodium pyruvate, 0.2 mM serine, 10% supplemented calf serum (SCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml hydrocortisone. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and air.

Focus assay

Focus assays were performed by plating 5 x 10^4 cells per 100 mm culture dish in culture medium as described, except that the medium

contained 5% fetal bovine serum (FBS) rather than 10% SCS. Normal foreskin-derived fibroblasts from a normal neonate were used as negative control cells in focus assays. Twenty four hours after plating, the medium was changed to medium identical to culture medium prepared as described except that it contained 1% FBS. Cells were examined microscopically each week for evidence of focus formation (a localized piling up of cells resulting from continued cell division). Some foci were picked and cultivated as individual cell strains. Other plates were stained four to six weeks after plating.

Tumorigenicity assay

The ability of cells to form tumors in mice was tested by injecting 1×10^6 to 10×10^6 exponentially growing cells (suspended in 0.2 ml Eagles medium with no additives) subcutaneously in the scapular region of six week old BALB/c athymic mice. In some instances cells were injected into collagen sponges that had been implanted subcutaneously at the injection site one week prior to injection of cells. The mice were examined weekly for tumors. Small nodules that occurred were excised, fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin prior to histological evaluation.

RESULTS

IM fibroblasts in culture

The fibroblasts that arose from biopsies of phenotypically normal skin of this IM patient appeared similar to normal human fibroblasts in cell culture. Cultures were passaged serially as described and eventually

the rate of growth slowed down and the cells ceased to divide at about 35 population doublings.

Foci from IM fibroblasts in 1% serum

Focus assays were performed to determine whether fibroblasts derived from phenotypically normal skin from this infantile myofibromatosis patient could form foci in low serum. Normal foreskin fibroblasts used as controls did not form foci in 1% FBS. However, fibroblasts from the infantile myofibromatosis patient consistently formed approximately two to four foci per 100 mm culture dish (Fig. 1). These results were observed in multiple culture dishes in two independent experiments. Microscopic examination revealed that the foci were composed of piled up spindle-shaped cells on a monolayer of spindle-shaped cells (Fig. 2). Cells were isolated from foci and were serially passaged in standard culture medium and were found to have a finite life span in culture.

IM fibroblasts form benign tumors in athymic mice

Early passage infantile myofibromatosis fibroblasts cell lines or focus-derived cell strains derived from infantile myofibromatosis fibroblasts were injected in athymic mice to determine whether such cells could form tumors in athymic mice. In the first of these tumorigenicity experiments, IM fibroblasts were injected into collagen sponges that had been implanted subcutaneously into two athymic mice one week prior to injection of cells. We used these collagen sponges because of reports that the sensitivity of the tumorigenicity assay is improved when cells are injected into collagen or some other matrix. Each mouse was implanted with two collagen sponges which resulted in a total of four injection sites in this experiment. One month after injection small nodules

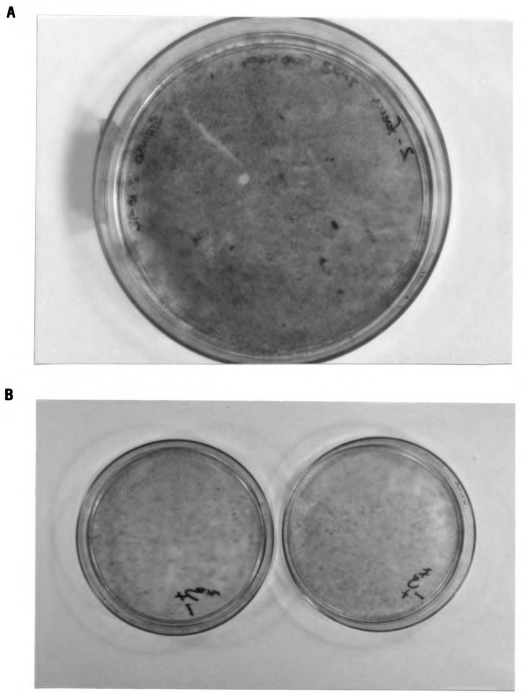


Figure 1. Focus formation of IM fibroblasts in 1% serum. (A) IM fibroblasts demonstrating dark staining foci. (B) Normal human foreskin fibroblasts that did not form foci.

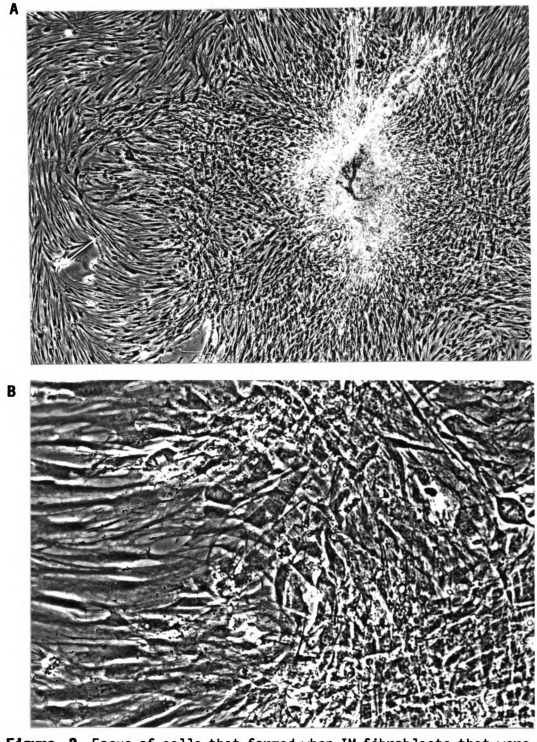


Figure 2. Focus of cells that formed when IM fibroblasts that were plated in medium containing 1% serum as described. (A) low power magnification demonstrating focus on background of non-focus forming cells. (B) high power magnification demonstrating dividing cells within focus.

persisted at the injection sites. One mouse was sacrificed and the tumors were removed from both sponge sites. Histological examination revealed what appeared to be neoplastic cells with remnants of collagen present in the tissue, but the presence of the remnants of collagen made histological diagnosis difficult. The tumors in the other mouse regressed within about one week after the first mouse was sacrificed.

The ability of IM fibroblasts to form small tumors in athymic mice was reassessed by injecting IM cell lines and focus-derived IM strains into athymic mice. In this experiment, no sponges were used and the mice were injected subcutaneously at only one site on each mouse (between the scapula). Two focus-derived infantile myofibromatosis strains were injected into a total of five athymic mice. The two early passage primary IM fibroblast lines were injected into three athymic mice each. We did not detect formation of nodules in any of the mice that were injected with focus-derived IM strains. Previously we have determined that when normal fibroblasts from several humans, including neonates, children, and adults, were injected into athymic mice, the swelling at the site of injection caused by the cells and medium persists only for seven to ten days (slightly longer when cells are injected into the collagen sponges) and tumors are never detected either with or without sponges. These results have been confirmed in more than 50 athymic mice (data not shown). Nodules did not persist beyond two weeks in the mice that were injected with focus-derived strains. However, small nodules persisted for three to four weeks in three of five mice that were injected with the primary IM fibroblasts cultures. One of the mice injected with a primary IM fibroblast strain was sacrificed after 15 days. Microscopic examination of a hematoxylin and eosin stained section from this nodule demonstrated that the nodule was composed cells identical to the cells that were

present in the nodules that occurred when IM fibroblasts were injected into sponges in athymic mice and identical to the cells of the tumor that was removed from this IM patient shortly after birth (Fig. 3).

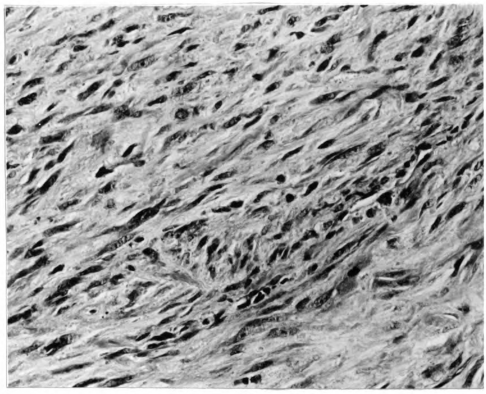
Discussion

Extensive experiments from this laboratory demonstrate that fibroblasts from normal skin of neonates, children, or adults do not form foci when assayed in 1% serum as described in this paper (J. McCormick, unpublished studies). The fact that this occurs repeatedly at a high frequency in fibroblasts obtained from apparently normal skin from a child with IM strongly suggests that some fibroblasts of this individual have abnormal growth characteristics. In our experiments we have found that expression of various growth factor related oncogenes can be responsible for such foci (see below). The fact that IM fibroblasts form fibromas in athymic mice is a clear indication that the IM cells, or at least a subpopulation of them, are neoplastic.

Since only a fraction of the cells in these IM fibroblast populations were capable of forming foci, i.e., approximately two to four distinguishable foci formed per 5×10^4 cells, injection of 1×10^6 to 10×10^6 cells into an athymic mouse would include 40 to 800 cells capable of focus formation. Based on experiments of various frankly malignant human mesenchymal cells studied in this laboratory (J. McCormick), it is very unlikely that less than 1×10^4 neoplastic cells, even in the presence of 1×10^6 to 10×10^6 carrier cells, could give rise to a tumor within a three month period. Therefore, it appears that non-focus forming IM fibroblasts that were injected along with the few cells capable of focus formation, somehow contributed to the formation of the nodules that

Figure 3. (A) Hematoxylin and eosin stained section of a tumor removed from IM patient shortly after patient was born. (B) Hematoxylin and eosin stained section of a tumor that occurred in an athymic mouse as a result of subcutaneous injection of IM fibroblasts as described.

A



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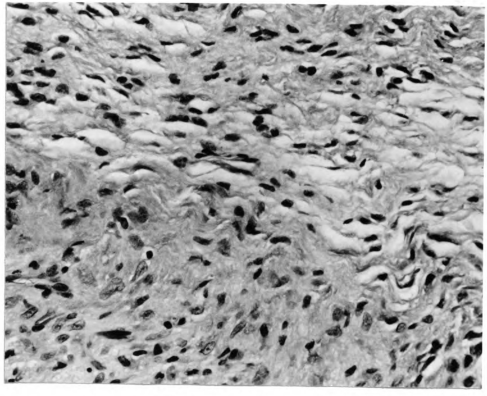

occurred in athymic mice. One possible explanation is that the cells capable of focus formation in low serum represent the cells that cause the nodules in mice, but that these focus forming cells can recruit other cells (non-neoplastic) in the formation of these small tumors. Recruitment of non-neoplastic cells in a neoplastic role occurs, for example, when neoplastic cells secrete a growth factor that causes non-neoplastic cells in the tumor environment to proliferate.

Cells derived from foci gave rise to strains that did not have any growth or morphological characteristics that distinguished them from the IM fibroblast lines from which they were derived, although they could not be extensively studied because of their short life span. The focus-derived strains stopped dividing within a few population doublings after being expanded to 10×10^6 to 20×10^6 cells and senesced in a typical pattern (10). Since clonally derived populations senesce 10-15 population doublings before bulk populations, this is not unexpected. This possibly explains why the focus-derived cells did not form nodules in athymic mice.

A number of experiments have shown that neoplastic, but non-malignant human cells can form benign tumors in athymic mice that eventually regress. We have observed that cells established in culture from benign human tumors, including several neurofibromas and a benign myxoma, form small nodules in athymic mice with a histological appearance typical of the tumor of origin (J.Wray, unpublished studies). These regress if not removed. Sheela et al. (11) reported similar results with human neurofibroma tissue. Yang et al. (15) demonstrated that when a human fibroblast strain was transfected with a v-*sis* oncogene, clonal populations expressing the v-*sis* protein formed fibromas in athymic mice that frequently regressed.

Experiments from this laboratory designed to transform human fibroblasts *in vitro* have generated cells with characteristics similar to the IM fibroblasts in this experiment. When an mutationally activated, over-expressed H-*ras* oncogene was transfected into normal diploid human fibroblasts, transfected cells formed foci in low serum, but the focus-derived cells failed to malignant tumors when they were injected into athymic mice (6). Similar experiments in which other oncogenes including v-*sis*, c-*sis*, (4,5), or N-*ras* (13) were transfected into normal human diploid fibroblasts produced similar results. When these same genes were transfected into an infinite life span human fibroblast cell strain, the cells expressing these genes were malignantly transformed (7,14). These results suggest that immortalization is a critical step in the transformation process and that cells that do not have this property may form lesions in athymic mice that fail to grow progressively and eventually regress.

It will be of interest to examine the cells in culture from myofibromas from this individual as well as others with myofibromatosis. We predict that all or most of these cells will grow to high density (i.e., like a focus) when in 1% serum, which is indicative of a defect in a growth control pathway. The underlying defect in such individuals can only be guessed at at this time. Since the underlying genetic defects that predispose individuals to neoplasia have inevitably been shown to encode a protein that functions as a suppressor gene (3), we assume that an IM individuals inherit a defective suppressor gene which controls fibroblast cell growth. In the course of normal development, the other allele is inactivated by chance mutational processes in some fibroblasts. Since background mutations rates in human cells are 1-100 per 10^7 cells per generation, many cells with such a phenotype would already be present in

a full term IM infant. This could account for the tumors seen at birth or later. However, if the situation were this simple, tumors would be expected to be far more common than they are in IM individuals. We postulate that IM fibroblasts that have lost the function of both copies of a suppressor gene also need the activation of an oncogene to form a fibroma. Such a model agrees better with studies carried out to model the carcinogenesis process in human fibroblasts in this laboratory (9).

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APPENDIX

APPENDIX

Establishment of cell lines from human soft tissue tumors

As part of my research, I established several cell lines from benign and malignant human soft tissue tumors. The table on the following page provides a summary of the results of this work. Those cell lines that are listed in bold letters are those that have been discussed in the text of this dissertation. Some basic information on each of the tumors and the cell line that resulted is listed in the columns. All of these cell lines were established in the Carcinogenesis Laboratory at Michigan State University with the exception of the three neurofibrosarcoma cell lines that were provided from Dr. Thomas Glover of the University of Michigan. Dr. Mark Zalupski, of Wayne State University, provided the majority of the human tumor material with the exception of the neurofibromas and the normal biopsies from the patient with infantile myofibromatosis.

The information that is given in this table includes the age and sex of the patient from which each tumor was obtained. Fresh tumor material was implanted into athymic mice in some instances and the results of these experiments are listed as *yes* if a tumor occurred and *no* if a tumor did not occur. The next column lists the results of experiments in which cells from culture were injected into athymic mice. Finally, the last column lists the results of continuous culture of each of the cell lines.

TUMOR CELL LINES

Cell Line Designation	Age of Patient	Sex of Patient	Tumor by Implant	Tumor by Cell Line	Population Doublings
Fibrosarcomas					
WSU-1	72	M	yes	yes	150 +
WSU-5	70	M	yes	no	30 ---> senesce
WSU-11			no	no	20 ---> senesce
WSU-13			not done	no	35 ---> senesce
WSU-14			not done	no	35 ---> senesce
WSU-16			no	no	
Leiomyosarcomas					
WSU-7	72	M	yes	not done	few --> senesce
WSU-12	55	F	yes	no	30 ---> senesce
WSU-15	64	M	not done	yes	35 ---> senesce
WSU-18	43	F	no	no	50 ---> senesce
Neurofibrosarcomas					
NF1-WSU-4	44	M	no	no	150 +
NF1-WSU-10	17	F	yes	yes	200 +
NF1-WSU-17	33	F	yes	yes	150 +
NF1-88.3[1]			not done	no	30 ---> senesce
NF1-88.14[1]			not done	no	30 ---> senesce
NF1-90.8[1]			not done	yes	100+
Other					
WSU-2	66	M	no	no	few --> senesce
WSU-3	70	F	yes	yes	30 ---> senesce
WSU-8			no	no	30 ---> senesce
WSU-9			no	no	40 ---> senesce
Neurofibromas					
NFb-1[2]	32	F	yes (regresses)	yes (regresses)	30 ---> senesce
NFb-2					
NFb-3					
NFb-4					
NFb-5					
NFb-6					
NFb-7					
NFb-8					
NFb-9					
NFb-10					
NFb-11					
NFb-12[3]					
Myofibromatosis					
TP-1	8	M	not done	yes (regresses)	50 ---> senesce
TP-2					

[1] = Cell lines provided by Dr. T. Glover of The University of Michigan.
[2] = NFb-1 through NFb-11 are from same 32 year old female with NF1.
[3] = NF1 patient of unknown sex and age. Tumor provided by Dr. Mark Lebwohl, New York.