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199

This is to certify that the

dissertation entitled

The Use of Nerve Growth Factor as a Reverse Transforming Agent for the Treatment of Tumors of Neurogenic Origin

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pathology

Major professor

Date July 30. 1991

THE USE OF NERVE GROWTH FACTOR AS A REVERSE TRANSFORMING AGENT FOR THE TREATMENT OF TUMORS OF NEUROGENIC ORIGIN

By

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A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Pathology

1991

ABSTRACT

THE USE OF NERVE GROWTH FACTOR AS A REVERSE TRANSFORMING AGENT FOR THE TREATMENT OF TUMORS OF NEUROGENIC ORIGIN

By

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The standard approach to cancer therapy generally involves selective removal or destruction of neoplastic cells by surgery, radiation or chemotherapy, or a combination thereof. However, because primary CNS tumors are often not amenable to surgical removal and generally tend to infiltrate the surrounding neuropile, novel therapeutic approaches may be necessary to augment traditional treatment strategies. One such option relies on the management of cancer through the control of differentiation and growth of neoplastic cells. The concept of reverse transformation proposes that certain agents may be able to activate pathways involved in differentiation, and in the process, suppress pathways essential for maintenance of the transformed phenotype.

Nerve growth factor (NGF), a compound important in the development, maintenance and regeneration of the mammalian sensory and sympathetic nervous systems, represents one such potential reverse transforming agent. Because NGF is able to stimulate the differentiation of a variety of normal and neoplastic cell lines of neurogenic origin, it may prove useful as a reverse transforming agent for the treatment of tumors of neuroectodermal origin.

This thesis chronicles research undertaken to investigate the reverse transforming potential of NGF. Two principal areas were studied. In the initial phase, a variety of tumor cell lines were evaluated for response to NGF *in vitro*. This was followed by an evaluation of the response of subcutaneous tumors implants to NGF in experimental animals. *In vitro* studies demonstrated the ability of NGF to induce neoplastic cells of neurogenic origin to develop a more differentiated phenotype, to diminish or arrest the growth rate of neoplastic cells in culture and to induce changes that persist in the absence of the compound. Subsequent *in vivo* tests demonstrated the ability of NGF to decrease the growth rate of tumor implants by 65% or more.

The ability of NGF to induce differentiation *in vitro* and diminish tumor growth rate *in vivo* indicates that nerve growth factor may have significant potential as a reverse transforming agent for the treatment of neurogenic tumors.

Dedicated to my parents, William J. Yaeger and Mary Ann Brink, and five wonderful sisters, Sharon, Susan, Teresa, Coleen and Anne, thank you for a lifetime of support. I would also like to dedicate this thesis to my wife, Susan, with love and appreciation.

ACKNOWLEDGEMENTS

I would like to extend a special thank you to Dr. Adalbert Koestner, my major professor, for his guidance, support and friendship. I am indebted to Dr. Koestner for his assistance and insights. His advice was invaluable during my program and shall continue to aid me in future endeavors.

I would also like to acknowledge the remaining members of my committee; Dr. Kathyrn Lovell, Dr. Keiji Marushige, Dr. Jon Patterson and Dr. Clifford Welsch. It would be difficult to find a nicer, more knowledgeable group with which to work and I truly appreciate their guidance and support.

I would like to thank Dorothy Okazaki, Dr. Yasuko Marushige, Cori Oakley and Ralph Common. Their technical support and assistance was invaluable.

I am grateful to all the members of the department of Pathology and AHDL. I wish to acknowledge their part in making my stay at Michigan State University a rewarding and enjoyable experience.

Lastly, I would like to thank my wife, Susan, for her understanding and ready assistance during my program. She was at once a wife, unpaid laboratory technician and assistant, and my major means of emotional support.

Thank you all. I shall always have fond memories of Michigan State.

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KEY TO ABBREVIATIONS

BID.....Twice-a-day
CNS.....Central nervous system
D54.....D54 mixed glioma cell line.
DMEM.....Dulbecco's modified eagle's medium
EGF.....Epidermal growth factor
ENU.....Ethylnitrosourea
FBS.....Fetal bovine serum
Hs294.....Hs294 malignant melanoma cell line
IP.....Intraperitoneal
MRI.....Magnetic resonance imagery
NGF.....Nerve growth factor
NGFR.....Nerve growth factor receptor
PC12.....PC12 pheochromocytoma cell line
PNS.....Peripheral nervous system
SID.....Once-a-day
TE671.....TE671 medulloblastoma cell line
U251.....U251 Glioblastoma multiforme cell line
U118.....U118 Glioblastoma multiforme cell line

INTRODUCTION

INTRODUCTION

The standard approach to cancer therapy generally involves selective removal or destruction of neoplastic cells by surgery, radiation or chemotherapy, or a combination thereof. However, novel therapeutic approaches may be able to augment these traditional treatment strategies. One such option is the management of cancer through control of differentiation and growth of neoplastic cells. Malignant transformation, or an integral event in malignant transformation, may be associated with an aberrant or incomplete response to normal effectors regulating differentiation and growth. If this were true, then specific differentiating agents might be able to stimulate neoplastic cells to differentiate and reverse many of the transformed properties of the neoplastic cells. Such a process is the antithesis of neoplastic transformation and is termed reverse transformation. It is based on the premise that certain agents may be able to activate processes which stimulate neoplastic cells to differentiate to a point where they no longer exhibit neoplastic behavior.

Nerve Growth Factor (NGF) represents one such potential reverse transforming agent. The capability of NGF to stimulate the differentiation of a variety of normal and neoplastic cell lines of neurogenic origin suggests that it

may prove useful as a reverse transforming agent. In particular, studies demonstrating the ability of NGF to retard cell proliferation and induce morphologic changes in anaplastic glioma cells (Marushige et al. 1987; Kumar et al. 1990), PC12 pheochromocytoma cells (Greene and Tischler, 1976) and neuroblastoma cells (Reynolds and Perez-Polo, 1975; Jensen 1987; Matsushima and Bogenmann, 1990) indicate that it is capable of reversing some of the transformed properties of neoplastic cells of neurogenic origin. In response to NGF, these tumor cell lines develop a more differentiated phenotype, and, in the process, lose the capacity for uncontrolled growth.

The literature review will outline the properties of NGF and the rationale behind its evaluation as a reverse transforming agent. Susequent chapters will describe studies designed to evaluate the reverse transforming potential of nerve growth factor.

LITERATURE REVIEW:

The discovery of nerve growth factor

The discovery of NGF was, like many in science, a fortuitous gift from an unexpected source: a mouse sarcoma. NGF was discovered as a result of the striking morphologic effects that it caused. When fragments of mouse sarcoma 37 or 180 were grafted to the body wall of 3-day chick embryos, sympathetic and sensory nerve fibers were stimulated to innervate the neoplastic tissue where they built an extraordinary network of fibers (Bueker 1948; Levi-Montalcini 1987). This extensive outgrowth was accompanied by an increase in the volume of corresponding ganglia of up to 6 times (Levi-Montalcini 1951). As the transplantation of the sarcomas to the chorioallantoic membrane elicited similar effects, it was hypothesized that the agent responsible for the induced changes was a soluble, diffusible product that was released from the sarcomas (Levi-Montalcini 1952; Levi-Montalcini and Hamburger, 1953). An *in vitro* assay confirmed this suspicion as sensory and sympathetic ganglia explanted from 8-day chick embryos and grown in a semisolid medium in proximity to fragments of mouse sarcoma 37 or 180 exhibited an eruption of nerve fibers of maximal density on the side facing the tumor (Levi-Montalcini et al. 1954). The majority

of this nerve outgrowth activity was identified in the microsomal fractions of these tumors (Cohen et al. 1954).

In another fortuitous discovery, snake venom was identified as possessing similar nerve growth promoting activity (Cohen and Levi-Montalcini, 1956; Levi-Montalcini and Cohen, 1956; Cohen 1959). This led directly to the evaluation of the murine homolog of the venom producing glands, the mouse submandibular salivary gland, for NGF activity (Cohen 1960). When added in minute quantities to the culture medium, homogenates of the mouse salivary gland elicited an even denser and more compact fibrillar halo (Cohen 1960; Levi-Montalcini and Booker, 1960b; Aloe et al. 1981). Though considerable confusion centered around why such diverse, non-neural sources should possess nerve growth promoting activity, it was nonetheless believed that this factor might represent an important clue to understanding the development and maintenance of the nervous system. The identification of this very rich source of NGF launched an extensive and systematic search into the compound's chemical structure, mechanisms and spectrum of action.

The nature of NGF

Murine NGF exists as a high molecular weight complex with a sedimentation coefficient of 7S and a molecular weight of approximately 130,000 (Varon et al. 1954). It consists of two alpha subunits, two gamma subunits, and a single, 2.5S beta

subunit (Smith et al. 1968; Greene et al. 1971). All of the neurotrophic activity resides in the beta subunit (Greene et al. 1971) which consists of a dimer of two identical chains of 118 amino acids (Angeletti and Bradshaw, 1971) held together by noncovalent bonds (Bocchini and Angeletti, 1969). The other subunits do not influence the activity of the beta, and the 7S must come apart and liberate the beta subunit before any activity can be expressed (Stack and Shooter, 1979). The dissociation of the high molecular weight complex into active and inactive subunits can be accomplished during the extraction procedure through the use of low pH and separation via ion-exchanges columns (Varon et al. 1954).

The active NGF molecule appears to be a highly conserved protein. Murine NGF elicits a strong response in avian, rat, hamster, monkey and human tissues. A comparison of mouse beta-NGF nucleotide and amino acid sequences with corresponding human sequences reveals high levels of homology (Ullrich et al. 1983). The nucleotide sequences are 86% homologous overall (Ullrich et al. 1983). Not only is the NGF molecule highly conserved, but extensive sequence homology also exists for the nerve growth factor receptor (NGFR). The NGFR has been cloned in the rat, chicken and human, and the three receptors, especially in the cysteine-rich binding site, share extensive sequence homology (Johnson et al. 1986a; Radeke et al. 1987; Large et al. 1989).

Properties of NGF

The regulation of neuronal development can be considered in terms of control of cell death, differentiation and orientation. NGF appears to play an important role in each of these areas in the peripheral nervous system. NGF plays a vital trophic role especially during early developmental stages. NGF enhances differentiation processes, such as neurite outgrowth, and guides the direction of growing or regenerating neurites along its concentration gradient (Gunderson and Barrett, 1980; Campenot, 1982a; Collins and Dawson, 1983).

Neurotrophism

Some of the most astonishing examples of the importance of NGF have been demonstrated through the use of anti-NGF-antibodies administered to populations of prenatal, neonatal and adult rodents. Anti-NGF-antibodies present during the neonatal period resulted in near total disappearance of sympathetic para- and prevertebral ganglia (Levi-Montalcini and Booker, 1960a; Levi-Montalcini and Booker, 1960b; Levi-Montalcini 1964; Otten et al. 1979; Johnson et al. 1980; Kessler and Black, 1980a) a process which was termed immunosympathectomy (Levi-Montalcini and Angeletti, 1966). When administered in utero, anti-NGF-antibodies not only resulted in the degeneration of the sympathetic nervous system but also in the degeneration of sensory neurons and chromaffin

cell precursors in the adrenal medulla (Aloe and Levi-Montalcini, 1979; Johnson et al. 1980). Even in adult animals, immunization against NGF results in a 30-40% cell loss in the superior cervical ganglion (Gorin and Johnson, 1980).

Additional information as to the importance of NGF was gained through the administration of supplemental NGF. Initial experiments in mice indicated that injection of crude NGF preparations into newborn mice resulted in a 4-6 fold increase in the size of the superior cervical ganglia (Levi-Montalcini and Booker, 1960b). Additional experiments further clarified the nature of this response. During normal development, there is a considerable decrease in the number of sympathetic neurons in the superior cervical ganglion. Administration of NGF to 6 day old rats prevented this decrease via a more rapid rate of neuronal maturation and the prevention of cell death (Hendry and Campbell, 1976; Hendry 1976; Zaimis 1971). This, combined with the fact that NGF levels in various target organs are correlated with the density of innervation (Holzbauer and Sharman, 1972; Campenot 1982b), suggests that the density of target organ innervation may be controlled by the level of NGF produced by the target organ. The ability of NGF to support the survival of neurons (neurotrophism) has also been demonstrated in studies involving lesion induced neuronal degeneration. Exogenous NGF administration prevented nerve cell death following

transection of NGF-dependent postganglionic axons of the superior cervical ganglia (Levi-Montalcini et al. 1975; Hendry 1975; Johnson et al. 1980; Johnson et al. 1986b). Additionally, chronic intracerebroventricular infusion of NGF in rats or primates with fimbria-fornix lesions prevents the degeneration of basal forebrain cholinergic neurons (Hefti 1986; Williams et al. 1986; Kromer 1987; Gage et al. 1988; Tuszynski et al. 1990).

Neurotropic effects

NGF can direct growing or regenerating axons of sensory and sympathetic fibers along its concentration gradient. These neurotropic effects have been established both in vitro and in vivo (Levi-Montalcini 1976; Menesini-Chen et al. 1978; Gunderson and Barrett, 1979; Gunderson and Barrett, 1980; Campenot 1982a). When NGF was injected into the brain of neonatal rats for 6-10 days, nerve fibers originating from sympathetic ganglia were observed to enter the spinal cord and ascend the dorsal columns to the site of injection in the brain stem (Levi-Montalcini 1976). In vitro, growth cones of chick sensory ganglia were observed to rapidly turn and grow towards the highest concentration of NGF when exposed to local concentrations of the growth factor (Charlwood et al. 1972; Letorneau 1978; Gunderson and Barrett, 1979; Gunderson and Barrett, 1980). Furthermore, NGF adsorbed in specific patterns onto a substratum can guide axonal growth along the

patterns, thereby exerting a haptotactic effect (Gunderson 1985). It is through such neurotropic effects that NGF may assist in the precisely oriented growth necessary for the formation of specific synaptic connections within the nervous system (Yanker and Shooter, 1982).

Maturation and differentiation

Maturation and differentiation are the two properties of NGF central to its reverse transforming potential. Much of the information in this area has been generated *in vitro* utilizing various neoplastic cell lines and will be discussed in detail in later sections. However, *in vitro* and *in vivo* studies involving non-neoplastic tissue offer compelling evidence that NGF is capable of stimulating maturation and/or differentiation. Administration of NGF can accelerate the maturation of sensory and sympathetic neuroblasts to essentially postmitotic neurons (Mobely et al. 1977; Kessler et al. 1979). Exogenous NGF is also capable of influencing the course of development of certain cell populations. Prenatal injection of NGF was shown to produce massive transformation of chromaffin cell precursors to sympathetic neurons (Aloe and Levi-Montalcini, 1979). This was confirmed *in vitro* as NGF caused a phenotypic shift in chromaffin cells (Unsicker et al. 1978; Aloe and Levi-Montalcini, 1979) resulting in their neuronal differentiation accompanied by a number of chemical, ultrastructural and morphologic changes

characteristic of neuronal rather than a neuroendocrine phenotype (Greene and Shooter, 1980).

NGF-induced down-regulation of mitogenic receptors

There is considerable evidence that NGF is capable of stimulating neurogenic cell lines to undergo differentiation (Waris et al. 1973; Reynolds and Perez-Polo, 1975; Greene and Tischler, 1976; Unsicker et al. 1978; Aloe and Levi-Montalcini, 1979; Greene and Shooter, 1980; Vinores and Koestner, 1981; Reynolds and Perez-Polo, 1981; Jensen 1987; Marushige et al. 1987; Marushige et al. 1989b; Kumar et al. 1990; Matsushima and Bogenmenn, 1990; Yaeger et al. 1991). In at least one cell line, this process is accompanied by changes that cause these cells to become less susceptible to mitogenic signals. Treatment of PC12 cells with NGF reduces the amount of epidermal growth factor (EGF) bound to the cells by 80% or more (Huff and Guroff, 1979; Huff et al. 1981). This decrease in binding is due to a reduction in the number of cell surface receptors caused by decreased receptor biosynthesis (Lazarovici et al. 1987). Such a process fulfills the criteria for heterologous down-regulation and may represent a coordinated change in the potential of the differentiated PC12 cell to interact with the hormonal environment (Lazarovici et al. 1987). This decrease in the availability of mitogenic receptors on the surface of the cells during differentiation may restrict the ability of the

differentiated neurons to proliferate (Lazarovici et al. 1987). Thus, as part of its differentiating response, NGF appears to down-regulate systems that have the potential to stimulate proliferative pathways. This may be a part of the mechanism whereby the differentiated phenotype is maintained.

NGF and the CNS

The role of NGF in the development and maintenance of peripheral sensory and sympathetic neurons has been well characterized. Studies indicate that NGF may also play a role in the development and maintenance of cholinergic neurons in the mammalian forebrain (Williams et al. 1986).

Initial evidence that NGF may play an active role in the CNS was demonstrated by the identification of both NGF and nerve growth factor receptors (NGFR) in the brain. The CNS has been shown to contain NGF mRNA, NGF antigen and NGFR (Greene and Shooter, 1980; Scott et al. 1981; Ayer LeLievre et al. 1983; Taniuchi and Johnson, 1985; Richardson et al. 1986). Nerve cells, especially those located in the hippocampus and cortical areas manufacture large quantities of NGF mRNA and NGF protein (Korsching et al. 1985; Shelton and Reichardt, 1986; Whittemore et al. 1986). Small and large neuronal populations located in different brain areas have been shown to exhibit all the properties and responses typical of sensory and sympathetic cells such as: 1) the presence of specific receptors (Szutowicz et al. 1976), 2) retrograde

transport of NGF (Seiler and Schwab, 1984), 3) increased neurotransmitter synthesis in response to NGF (Gnahn et al. 1983; Hefti et al. 1984; Mobley et al. 1985) and 4) a trophic response characterized by prevention of cell death by exogenous NGF (Williams et al. 1986; Kromer 1987). Reports indicate that the majority of NGFR in the adult rat and monkey brain is found in the cholinergic neurons and that NGF has a neurotrophic effect on these cholinergic neurons (Schwab et al. 1979; Seiler and Schwab, 1984; Taniuchi and Johnson, 1985; Kordower et al. 1988; Batchelor et al. 1989). Chronic intracerebroventricular infusion of NGF in rats or primates with fimbria-fornix lesions prevents the degeneration of basal forebrain cholinergic neurons (Hefti 1986; Williams et al. 1986; Kromer 1987; Tuszynski et al. 1990).

One of the motivating factors behind research into the effects of NGF in the CNS centers around the well characterized neurotrophic properties of this compound in both the CNS and PNS. It is theorized that the neurotrophic properties of NGF may prove useful in preventing or limiting the severity of a number of neurologic disorders characterized by degeneration of selected neuronal populations such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and others. (Appel 1981).

The nerve growth factor receptor

In the previous sections mention was made of the nerve growth factor receptor (NGFR). The receptor is a central issue in any discussion of the effects of NGF because the growth factor must first bind to specific cell surface receptors in order to effect a response. Though this point is incontrovertible, the exact nature of the receptor for NGF and the NGF-NGFR interaction is unclear. The nature of the receptor and the character of this interaction has recently been clarified to a greater degree (Buxser et al. 1990; Hempstead et al. 1991), however, the mechanisms by which this complex triggers changes within the cell have not, as of yet, been fully elucidated.

A wide variety of cell types possess NGFR. These include sensory and sympathetic neurons (Verge et al. 1989), cholinergic CNS neurons (Cohen-Cory et al. 1989), meningeal cells (Rettig et al. 1987), glial cells (Rettig et al. 1987), Schwann cells (Zimmerman and Sutter, 1983; Taniuchi et al. 1986; Rettig et al. 1987), adrenal chromaffin cells, and the neoplastic counterparts of many of these cell types including neuroblastomas (Rettig et al. 1987; Marchetti et al. 1987; Chen et al. 1990), medulloblastoma, pheochromocytoma (Landreth and Shooter, 1980), gliomas (Kumar et al. 1990), neurinomas and melanomas (Fabricant et al. 1977). Cells of hematopoietic lineage, mast cells and B-lymphocytes, also express NGF-R (Thomson et al. 1988).

The nerve growth factor receptor is generally regarded to be a 70-80 kDa phosphorylated glycoprotein (Hosang and Shooter, 1985; Chao et al. 1986; Green and Greene, 1986; Ross et al. 1987). NGF binds to specific NGFR located on the nerve endings of sympathetic and sensory fibers by a selective and saturable mechanism, is internalized and transported retrogradely to the corresponding perikarya where it evokes its characteristic biochemical effects (Hendry et al. 1974; Stockel et al. 1974; Duman et al. 1979; Thoenen and Barde, 1980; Hamburger et al. 1981; Schwab et al. 1982; Hosang and Shooter, 1985; Johnson et al. 1987). NGF, when retrogradely transported to the neuronal soma will assure maintenance of the differentiated state of the neuron (Hendry 1976; Thoenen and Barde, 1980). The NGF-NGFR complex is internalized in vesicles in a fashion similar to that observed for EGF and insulin (Schlessinger et al. 1978; Levi et al. 1980). Following internalization and retrograde transport, it is the association of NGF and its receptor with the nucleus that closely parallels the effects of the hormone on neurite outgrowth (Yanker and Shooter, 1982). Interruption of retrograde axonal transport by surgical or chemical procedures has the same effect as the neutralization of endogenous NGF by specific antibodies (Thoenen and Barde, 1980; Harper and Thoenen, 1981; Schwab et al. 1982).

As with most polypeptide hormones and growth factors, the receptor plays an essential role in signal transduction. This

has been demonstrated by the fact that NGF injected directly into the cell cytoplasm has no effect on responsive cells. NGF injected into the cytoplasm of either naive PC12 cells or cells pretreated with NGF, did not induce process outgrowth compared with non-injected PC12 cells in the same culture dishes, despite the obvious responsiveness of the non-injected sister cultures to extracellularly applied NGF (Seeley et al. 1983).

Reports indicate that the binding of NGF to its specific surface receptor is inconsistent with a simple, single step interaction of NGF with its receptor (Sutter et al. 1979; Landreth and Shooter, 1980; Olender and Stach, 1980; Schechter and Bothwell, 1981; Tait et al. 1981; Bernd and Greene, 1984; Godfrey and Shooter, 1986; Green et al. 1986; Woodruff and Neet, 1986; Marchetti and Perez-Polo, 1987). This is indicated by a deviation from linear of Scatchard plots and the evaluation of association and dissociation kinetics. These types of analyses, along with the evaluation of physical properties, support the existence of at least two distinct classes of NGF-binding sites in neurons and PC12 cells (Sutter et al. 1979; Olender and Stach, 1980; Landreth and Shooter, 1980; Schechter and Bothwell, 1981; Tait et al. 1981; Sonnenfeld and Ishii, 1982; Stach and Wagner, 1982; Block and Bothwell, 1983; Vale and Shooter, 1983; Bernd and Greene, 1984; Godfrey and Shooter, 1986; Green et al. 1986; Green and Greene, 1986; Woodruff and Neet, 1986; Marchetti and Perez-

Polo, 1987; Kumar et al. 1990; Welcher et al. 1991): 1) a slow dissociation, high-affinity, trypsin resistent form that has a Kd of 0.2 nM in PC12 cells and a Kd of 10 pM in sensory and sympathetic neurons and is resistent to extraction with low concentrations of Triton X-100 and 2) a rapidly dissociating, low-affinity, trypsin sensitive form that has a Kd of 5 nM in PC12 cells and a Kd of 1 nM in sensory and sympathetic neurons and is extracted with low concentrations of Triton X-100. The high affinity receptors appear to be responsible for NGF internalization and for the differentiating response in PC12 cells (Green et al. 1986). The biologic response in neuronal cells is also thought to correlate with occupancy of the high affinity receptor.

Though reports describe two different NGF-binding sites, data indicates that a single protein is responsible for both the high-affinity and low affinity forms of the NGFR (Buxser et al. 1985; Green and Greene, 1986) with indirect evidence suggesting that the high-affinity NGFR consists of the low-affinity receptor complexed with an additional cytoplasmic or membrane protein that is necessary for signal transduction (Marchetti et al. 1987; Hosang and Shooter, 1987; Hempstead et al. 1989). Experiments in which receptors were labeled with ^{125}I-NGF, crosslinked and then immunoprecipitated with an antireceptor antibody, demonstrate the existence of two receptor proteins, an 80Kd species and a 200 Kd species. There appears to be structural homology between the 200-and

80-kDa receptor protein and it is suggested that the 200-kDa NGF binding protein is an oligomer, possibly a dimer, of the 80-kDa NGF binding protein (Buxser et al. 1985). It has been proposed that low affinity, rapidly dissociating binding of NGF results when NGF associates with a single 80-kDa receptor molecule and that the high affinity NGF binding results when each monomer of NGF binds to a separate receptor molecule, forming a dimeric receptor structure bridged by dimeric beta-NGF creating the 200-kDa complex (Buxser et al. 1985).

Removal of most of the intracellular portion of the low-affinity NGF-R has no effect on NGF binding to the extracellular domain of the receptor (Welcher et al. 1990). Furthermore, within the extracellular domain, the four cysteine-rich sequences when expressed alone are capable of binding NGF (Welcher et al. 1990). It has also been shown that the NGFR binds the homologous neurotrophic factor, brain-derived neurotrophic factor (Rodriguez-Tebar et al. 1990).

At this point, it becomes necessary to examine some puzzling concepts concerning the NGFR. It appears that a single protein is responsible for both high and low affinity binding and that these two forms exhibit interconversion (Vale and Shooter, 1982; Block and Bothwell, 1983; Buxser et al. 1983a; Buxser et al. 1983b). Yet if this were true, it is difficult to imagine how one form could be trypsin resistent and the other trypsin sensitive, considering that both must be present on the cell surface in order to interact with NGF.

There is also difficulty reconciling questions concerning the function of the numerous low affinity receptors. It has been suggested that because the low affinity sites are present in numbers at least 10-fold larger than high-affinity sites (Sutter et al. 1979; Claude et al. 1982), these low affinity receptors may be significant in terms of the absolute numbers of sites occupied. A spare receptor system (Stephenson 1956) might be involved, in which case a maximum response is attainable with occupation of only a fraction of the available sites. Even more puzzling is that some cell populations appear to respond in the apparent absence of high-affinity receptors and that lysis of PC12 cells results in the rapid loss of the high-affinity sites (Buxser et al. 1983b) apparently due to their rapid conversion to low-affinity sites after cell lysis (Buxser et al. 1983b).

Recent findings have helped to clarify some of these puzzling aspects of the NGFR. Innovative experimental approaches to the NGF-NGFR interaction have shed new light on the understanding of this association. These results do not contradict data obtained in previous studies, but rather offer new insight into the interpretation of existing information.

A major complication in the interpretation of receptor affinity data is the internalization of the NGF-NGFR complex at physiological temperatures. Scatchard plots, which are the most commonly used method to detect the two different binding affinities, are affected by internalization (Buxser et al.

1990). Therefore, it becomes essential to distinguish between high-affinity receptors and internalization. Using new approaches to try and separate these two events, Buxser et. al. (1990) addressed some of the aforementioned problems. The conclusions from their studies are rather insightful in light of existing data.

It does not appear that the heterogeneity of NGF binding arises from the presence of two different species of receptors. Rather, the identification of receptors with different binding affinities appears to be a consequence of the pathway by which the NGF-receptor complex is processed in the cell (Buxser et al. 1990). The thermodynamics of binding of ^{125}I-NGF to PC12 cells are only consistent with the initial formation of a single type of cell surface NGF-receptor complex consistent with the previously described low-affinity binding (Buxser et al. 1990). A delay of approximately 60 sec was observed after addition of ^{125}I-NGF before significant high-affinity receptor binding was detected (Buxser et al. 1990). This second type of site can be explained by the internalization of the ^{125}I-NGF complexes (Buxser et al. 1990). The previously described high-affinity complexes are not distinguishable from internalized NGF-receptor complexes and the dissociation from slow receptors is not significantly different from the return of internalized receptor to the cell surface (Buxser et al. 1990). These results provide a relatively simple model to explain binding of NGF to its

receptor and subsequent internalization (Buxser et al. 1990). The data are consistent with the presence of a single class of binding sites on the surface of PC12 cells (Buxser et al. 1990). The biologically relevant sites have an affinity of approximately 0.5 nM, can be internalized by the cells and require only a low level of occupancy, albeit for a long period of time, to initiate activation of the neurite outgrowth process (Buxser et al. 1990). This behavior is similar to that of the transferrin receptor (Chvatchko et al. 1984) and similar phenomena have been described for insulin (Chvatchko et al. 1984). It would appear that high-affinity binding has been confused with the internalization of the NGF-NGFR complex (Buxser et al. 1990). These results are consistent with much of what has been described about the NGF-NGFR interaction and provide a relatively straight forward, unifying concept for the NGF-NGFR association.

This had been the most recent and insightful information published on the subject. However, since the article by Buxser et. al. (1990), an entirely new concept has been unveiled. Recent studies (Hempstead et al. 1991) suggest that high-affinity NGF binding requires coexpression and cooperation between the low-affinity NGFR and the tyrosine kinase trk gene product (Hempstead et al. 1991).

The trk proto-oncogene encodes for a 140-Kd, membrane-spanning protein tyrosine kinase (p140-prototrk). Although trk was discovered as a rearranged oncogene from a colonic

carcinoma (Martin-Zanca et al. 1986), normal trk expression in vivo is limited to neural crest-derived sensory neurons (Martin-Zanca et al. 1990a; Martin-Zanca et al. 1990b). The p140-prototrk is intriguing because it is active in a select subset of neural crest derived neurons and appears to be the most exquisitely regulated mammalian tyrosine kinase that has been described (Barinaga 1991).

Cross-linking studies indicated the existence of two types of NGF receptors (Hosang and Shooter, 1985). Cross-linking of ^{125}I-NGF to its receptor with the photoactivatable agent N-hydroxylsuccinimidyl-4-azidobenzoate (HSAB), generated crosslinked complexes of 100K and 158K (Hosang and Shooter, 1985). The 158K crosslinked complex was generated only in cells that had high-affinity binding sites (Hempstead et al. 1991) and was considered to be the high-affinity receptor. These receptors were distinct from the previously characterized low affinity NGF receptors (p75-NGFR) (Chao et al. 1986; Johnson et al. 1986a; Radeke et al. 1987), which were represented by the 100K band. The 158-kD species cross-linked to NGF in PC12 cells and sympathetic neurons (Massague 1981; Hosang and Shooter, 1985; Buxer et al. 1985; Green and Greene, 1986) was not recognized by antibodies to p75-NGFR (Hempstead et al. 1990; Meakin and Shooter, 1991). The p75-NGFR did not appear to mediate a biologic response (Johnson and Taniuchi, 1987; Hempstead et al. 1988) following ligand binding and generated only low-affinity NGF-binding sites with

a Kd of 10^{-9} M (Chao et al. 1986). The generation of both high- and low-affinity sites required an additional factor besides the p75-NGFR.

The identification of this "additional factor" has long eluded investigators. Two recent studies have lead investigators to believe that the high affinity receptor was a tyrosine kinase. Maher (1988) demonstrated that NGF induced rapid phosphorylation of tyrosine residues when added to NGF-responsive PC12 cells while Meakin and Shooter (1991) demonstrated that the high affinity NGF receptors could be immunoprecipitated with anti-phosphotyrosine antibodies.

This high-affinity receptor expressing tyrosine kinase activity exhibited a molecular weight reminiscent of members of the trk family of tyrosine kinases (Barbacid et al. 1991). HSAB crosslinking experiments firmly identified the 158K species as the p140-prototrk crosslinked to NGF (Kaplan et al. 1991a). Following cross-linking to ^{125}I-NGF, the 158-kD species was immunoprecipitated with antibodies to NGF or p140-prototrk (Kaplan et al. 1991b). The immunoprecipitation of the 158-kD species was blocked by addition of the peptide from the p140-prototrk (Kaplan et al. 1991a) used to generate the antibody and was not seen if excess unlabeled NGF was added to the cells prior to cross-linking (Kaplan et al. 1991b).

Additional evidence that p140-prototrk is a receptor for NGF come from physiochemical studies. Picomolar concentrations of NGF stimulate p140-prototrk

autophosphorylation (Kaplan et al. 1991a). The stimulation of p140-prototrk tyrosine phosphorylation in response to addition of NGF to PC12 cells is rapid and specific, and occurs in the presence of physiological amounts of NGF (Kaplan et al. 1991a).

Membrane fusion studies clarified the contributions of p140-prototrk and p75-NGFR in the formation of the high affinity receptors. Both p140-prototrk and p75-NGFR exhibit binding characteristics of low-affinity NGFR (Kaplan et al. 1991b). PAE1c cells express only p75-NGFR and rtrk-3T3 cells express only the p140-prototrk. Scatchard analysis indicates that both these cell lines exhibit receptors with a single binding affinity consistent with low-affinity binding. However, if the membranes from these two cell lines were fused, they exhibited both high- and low-affinity binding (Hempstead et al. 1991).

Thus it appears that coexpression of proto-oncogene p140-prototrk and p75-NGFR are required for the formation of high-affinity NGF-binding sites (Hempstead et al. 1991). Experiments (Hempstead et al. 1991) confirm that two distinct protein species can bind NGF, and support earlier biochemical data describing the different proteolytic susceptibility, pH sensitivity and Triton X-100 solubility of the two kinetic forms of the receptor (Schechter and Bothwell, 1981; Vale and Shooter, 1982; Block and Bothwell, 1983; Buxser et al. 1983a). Results (Hempstead et al. 1991) provide strong support for the

hypothesis that the high affinity NGF binding site is composed of at least two NGF-binding components: the trk gene product and p75-NGFR.

These findings do not conclusively demonstrate that trk alone can trigger the specific biochemical pathways required for neuron survival and differentiation. They do greatly clarify the nature of the nerve growth factor receptor and the NGF-NGFR interaction. However, the specific biochemical pathways regulated by NGF remain to be elucidated.

Reverse transformation

The previous sections provide background information on the properties of NGF and the interaction with its receptor. Upcoming sections will detail the rationale behind the evaluation of NGF as a reverse transforming agent. However, before evidence detailing the ability of NGF to induce reverse transformation is presented, it would be beneficial to discuss the conceptual basis for this type of therapy and the crucial attributes of a reverse transforming agent.

Alterations or mutations in the genetic information are considered to be crucial in the process of neoplastic transformation. The therapeutic approach to eliminate these transformed cells generally involves surgical removal of the offending cells or their selective destruction through the use of radiation or chemotherapy. Since reverse transforming agents do not directly destroy neoplastic cells and can not

repair the genetic defect(s) present in these cells, how then can such agents restrain the malignant behavior of neoplastic cells? An insight into the answer to this question can be obtained from knowledge of normal cellular physiology. Terminally differentiated cells and their proliferation-competent precursors possess identical genetic make-up. However, one of these populations continues to proliferate while the other is restrained in a non-proliferating state. These two populations differ, not in their genetic content, but in the suppression or expression of genetic information by epigenetic factors. Herein lies the key to the potential of reverse transforming agents. Though reverse transforming agents are not able to repair the genetic abnormalities in transformed cells, they may be able to suppress pathways that are essential for the maintenance of the transformed phenotype. Just as epigenetic factors are able to maintain a cell in a terminally differentiated state, epigenetic mechanisms may also be able to persistently suppress the expression of the transformed phenotype in neoplastic cells.

The ability of epigenetic factors to modulate the expression of malignant characteristics has been demonstrated both *in vitro* and *in vivo*. When the B16 mouse melanoma cell line was implanted subcutaneously into susceptible animals, tumors developed rapidly and consistently. However, when B16 mouse melanoma cells were implanted into embryonic mouse skin at the time when pre-melanocytes migrate into the skin, a

significant decrease in tumor incidence was noted (Gerschenson et al. 1986). When conditioned medium from embryonic skin cultures was incubated with these same cells in culture, the growth of the melanoma cells ceased, the cells acquired a more normal morphology, and these cells failed to proliferate when placed in fresh growth medium (Gerschenson et al. 1986). In a similar study, significantly fewer tumors developed from C-1300-3 neuroblastoma cells that had been implanted into the second somite of neurula stage embryos than developed from untreated control cultures of the neuroblastoma line (Podesta et al. 1984). Thus, it appears that factors normally present in the microenvironment during development are able to suppress the malignant characteristics of certain neoplastic cell lines.

Factors in the microenvironment that may contribute to the suppression of the malignant characteristics include: 1) the properties of the extracellular matrix, 2) communication with neighboring cells and 3) the presence of soluble hormones or growth factors. Our research will concentrate on the ability of nerve growth factor, a growth factor with differentiating capabilities, to suppress neoplastic behavior.

A problem arises when striving to evaluate the response of neoplastic cells to this type of therapy. The mechanisms responsible for neoplastic transformation have not been elucidated for the cell lines under study. As such, it becomes difficult to demonstrate that the reverse transforming

agent has suppressed the expression of pathways responsible for transformation. Nonetheless, a set of crucial attributes essential for reverse transformation can be formulated which provide guidelines by which potential reverse transforming agents can be judged. These criteria are based on the goals of cancer therapy and the general properties of transformed cells. Once these criteria are defined, it becomes easier to appreciate the rationale behind the use of NGF as a reverse transforming agent and the research strategy utilized to evaluate the therapeutic potential of this compound.

As stated in the introduction, reverse transformation could be described as the antithesis of neoplastic transformation and characterized as the process whereby neoplastic cells are stimulated to differentiate to a point where they no longer exhibit neoplastic behavior. There are many different mechanisms by which a cell can undergo neoplastic transformation. It is likely that there are also a number of ways to partially or completely reverse this process. Though the mechanisms may vary, criteria vital for reverse transformation can be readily conceptualized.

One essential component of reverse transformation is suppression or cessation of the growth of neoplastic cells. A hallmark of neoplasia is growth that exceeds and is uncoordinated with that of normal tissues. This is not the result of neoplastic cells behaving as pathologic dynamos with greatly reduced doubling times and a large proportion of cells

in the growth fraction. In fact, the replication rate of neoplastic cells is generally less than that of many normal renewing tissues. Rather, tumors grow progressively because the fine homeostasis between cell growth and cell loss is disrupted. Therefore, as a characteristic of neoplastic cells is growth that exceeds that of normal tissue, it is vital that reverse transforming agents curb this unrestrained growth.

Since neoplastic cells are not directly destroyed during the process of reverse transformation, another vital characteristic of a reverse transforming agent is that the induced effects must persist. If the impact of an agent were transient and the neoplastic cells retained the ability for uncontrolled growth once the effects of the compound abated, then the patient would be no nearer a cure than before therapy, and the therapy would be of little value. Therefore, another essential feature of an agent that does not directly eliminate neoplastic cells during the course of treatment is persistence of the induced effects.

The third characteristic of a reverse transforming agent is the development of a more differentiated phenotype. Conceptually, this property does not appear to be as crucial as the previous two. If therapy fulfills the first two criteria, i.e., brings about a persistent arrest of tumor growth, then a significant therapeutic benefit would be gained, without the necessity for development of a more differentiated phenotype. However, the development of a more

differentiated state is generally the means by which a persistent suppression of tumor growth is attained. The vast majority of transforming agents are mutagens. Conversely, agents capable of inducing reverse transformation are generally compounds that promote differentiation. Indeed, differentiation therapy is essentially a synonym for reverse transformation. The establishment of a more differentiated state can result in reverse transformation either through terminal differentiation, in which the development of a differentiated phenotype is accompanied by cessation of growth, or through differentiation to a point where the neoplastic cells are once again brought under control of normal homeostatic mechanisms.

In conclusion, the basis for this type of therapy relies on the capacity of the reverse transforming agent to activate epigenetic mechanisms that persistently suppress the expression of the transformed phenotype. Three characteristics are of primary concern in evaluating the response of neoplastic cells to a reverse transforming agent: 1) the development of a more differentiated phenotype, 2) growth suppression or arrest and 3) persistence of the induced changes. An appreciation for these key principles will facilitate the analysis of data from <u>in vitro</u> and <u>in vivo</u> experiments which provide the basis for evaluating NGF as a reverse transforming agent.

In vitro studies

Information presented in this section describes results of *in vitro* experiments that demonstrate the ability of NGF to reverse several of the transformed properties of neoplastic cell lines of neurogenic origin. These studies indicate that, in a defined *in vitro* environment, NGF is able to induce several of the changes described as essential for reverse transformation.

Some of the most intriguing evidence for the reverse transforming ability of NGF comes from studies involving the PC12 pheochromocytoma cell line. As has already been mentioned, NGF is able to stimulate adrenal chromaffin cells (the cell type from which pheochromocytomas are thought to arise) to develop a number of chemical, morphologic and ultrastructural changes characteristic of a neuronal rather than neuroendocrine phenotype (Unsicker et al. 1978; Aloe and Levi-Montalcini, 1979; Greene and Shooter, 1980). NGF is able to stimulate similar changes in PC12 cells. PC12 adrenal medullary pheochromocytoma cells, when grown in the absence of NGF share many properties of adrenal chromaffin cells (Greene and Tischler, 1976; Greene and Rein, 1977). Within several days after the cells are exposed to physiologic levels of NGF, they begin to acquire a number of phenotypic properties of sympathetic neurons. These include initiation of neurite outgrowth, cessation of cell division and the development of electrical excitability (Greene and Tischler,

1976; Greene and Rein, 1977; Dichter et al. 1977; Schubert et al. 1977; Tischler and Greene, 1978; Rudy et al. 1982; Reed and England, 1986). Within 2-3 weeks, PC12 cells in the presence of NGF form an intricate network of electrically excitable neurites that can then form functional synapses with co-cultured muscle cells (Schubert and Whitlock, 1977).

The effects of NGF on the growth of PC12 cells warrants additional comment. The NGF-induced differentiation of PC12 cells involves the transition from a mitotic to a nonmitotic state (Lazarovici et al. 1987). It has been reported that if NGF was added to the culture medium immediately following removal of serum, the cells underwent approximately one doubling time before cessation of growth. (Greene and Tischler, 1976; Mobley et al. 1977; Greene 1978; Unsicker et al. 1978; Aloe and Levi-Montalcini, 1979; Greene and Shooter, 1980; Greene et al. 1983).

Additionally, reports (Huff et al. 1981; Lazarovici et al. 1987) have shown that exposure of PC12 cells to NGF caused down-regulation of epidermal growth factor (EGF) receptors by approximately 50% after 3-4 days (Rudkin et al. 1989). This heterologous down-regulation would appear to be an efficient means of desensitizing the cell to proliferative stimuli, thereby amplifying the differentiative pathways. (Lazarovici et al. 1987) Experiments involving the PC12 cell line demonstrate the ability of NGF to reverse some of the transformed properties of neoplastic cells of neurogenic

origin. The ability to transform a rapidly dividing population of neoplastic neuroendocrine cells into a non-dividing, functional population that resembles mature neurons illustrates the reverse transforming potential of NGF.

The differentiating effects of NGF are by no means restricted to PC12 cells. A variety of neurogenic cell lines respond in a similar manner. NGF reverses several of the transformed properties of a number of tumor cell lines of glial origin. The F98 anaplastic glioma cell line develops a more differentiated phenotype in response to NGF manifested by restoration of surface inhibition, a decrease in nuclear-cytoplasmic ratio and an increase in processes formation (Vinores and Koestner, 1980; Vinores and Koestner, 1981). NGF treatment also diminishes the growth rate of F98 cells in culture (Vinores and Koestner, 1980). The C6 glioma cells respond to NGF by becoming rounded and developing processes that form an interconnecting lattice or network between cells (Kumar et al. 1990). Eventually, cell proliferation of the C6 glioma line becomes arrested (as measured by ^{3}H-thymidine uptake assays) in response to NGF (Kumar et al. 1990). NGF treatment clearly retards cell growth of T9 anaplastic glioma cells (Marushige et al. 1987). T9 cells treated with NGF also develop a number of morphologic changes consistent with a more differentiated phenotype. NGF-treated cells become flattened and extend numerous cytoplasmic processes. (Marushige et al. 1987) The NGF-induced cytoskeletal changes in T9 cells

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support the concept that NGF is capable of morphological differentiation of anaplastic glioma cells endowed with NGF receptors (Marushige et al. 1989a).

As NGF plays an important role in the development and maintenance of the sensory and sympathetic nervous systems, it would seem reasonable that neoplastic cell lines derived from these cell types, such as neuroblastomas, a tumor of probable sympathetic origin, would be responsive to NGF. Indeed, several neuroblastoma cell lines have been shown to develop changes suggestive of neuronal differentiation in response to NGF (Waris et al. 1973; Reynolds and Perez-Polo, 1981; Marushige et al. 1987).

A number of human and murine neuroblastoma cell lines express NGFR, bind NGF, differentiate, and extend neurites in response to NGF (Perez-Polo et al. 1979; Otten et al. 1980; Kessler and Black, 1980b; Kessler and Black, 1981; Reynolds and Perez-Polo, 1981; Burmeister and Lyser, 1982; Sonnenfeld and Ishii, 1982). Cultured neuroblastoma cells tend to be round and do not produce processes (Waris et al. 1973). However, when these cells were grown in a medium containing NGF, they were stimulated to extend processes (Waris et al. 1973). SY5Y neuroblastoma cells extended processes exceeding 100 um in length after 3 days of NGF exposure (Perez-Polo et al. 1979; Sonnenfeld and Ishii, 1982). By 5 weeks, SY5Y cultures grown in the presence of 7S NGF exhibit a high degree of morphologic differentiation (Jensen 1987). The NGF-

induced neurites in SH-SY5Y cells had an ultrastructure similar to that of developing sympathetic ganglion cells and ended in structures typical of neuronal growth cones (Burmeister and Lyser, 1982).

The development of electrical excitability provides additional evidence that NGF is able to stimulate cultured neuroblastoma cells to develop properties indicative of neuronal differentiation. SY5Y and KA, human neuroblastoma clones, have the appearance of undifferentiated neuroblasts and are electrically unexcitable (Kuramoto et al. 1977). Treatment of these cells with NGF over a 5-day period results in the cells assuming morphologic characteristics of differentiated neurons (Perez-Polo et al. 1982) and the development of electrical excitability when monitored intracellularly (Kuramoto et al. 1981) or pharmacologically (Perez-Polo et al. 1982). These differentiating effects were identical whether the NGF was human or murine in origin (Perez-Polo et al. 1982).

NGF is able to stimulate neuroblastoma cells to develop a more differentiated phenotype and electrical excitability. These morphologic and functional changes are accompanied by a virtual arrest in cell division of SY5Y neuroblasts (Perez-Polo et al. 1979; Perez-Polo et al. 1982; Sonnenfeld and Ishii, 1982).

NGF appears to be able to induce at least two of the changes described as essential for reverse transformation.

Specifically, NGF is able to stimulate the development of a more differentiated phenotype and cause suppression of cell proliferation. These effects have been demonstrated in a wide variety of neurogenic tumor cell lines. However, evidence must be presented that demonstrates the ability of NGF to fulfill the remaining essential characteristic of a reverse transforming agent: the ability to induce effects that are persistent. Since neoplastic cells are not directly destroyed during the process of reverse transformation, it is vital that the differentiating effects persist.

In vitro experiments indicate that the morphologic changes induced by NGF persist in the absence of the compound. (Waris et al. 1973; Reynolds and Perez-Polo, 1981; Marushige et al. 1987) This has been demonstrated with neuroblastoma and anaplastic glioma cell lines (Waris et al. 1973; Reynolds and Perez-Polo, 1981; Marushige et al. 1987). T9 anaplastic glioma cells treated with NGF for 2 days undergo further morphologic changes during the succeeding 2 days in the absence of NGF and exhibit a pattern of cell growth and cell morphology similar to those grown in the continuous presence of NGF (Marushige et al. 1987). Cells grown in NGF for 2 days followed by 4 days without NGF, cells grown in NGF for 4 days and then for 2 days without and cells grown in the continuous presence of NGF for 6 days all show essentially identical morphological characteristics and growth pattern (Marushige et al. 1987). Thus, it appears that the effects of NGF

persist in its absence. Experiments involving a neuroblastoma cell line also demonstrate that the effects of NGF treatment are quite persistent as the NGF-induced morphologic changes continued to persist for as long as three weeks after switching to fresh medium (Reynolds and Perez-Polo, 1981).

The previous paragraph emphasizes a distinctive asset of NGF: the persistent nature of the response. The ability to induce a more differentiated phenotype is not unique to this growth factor. A variety of chemical agents, primarily analogs of second messengers, also possess this capability (Perez-Polo et al. 1982; Marushige et al. 1989b). Second messenger analogs are able to mimic not only the morphologic changes induced by NGF but are also capable of diminishing the growth rate. However, a crucial difference exists between these agents and NGF in terms of reverse transforming potential. Second messenger analogs, at least those tested to date, do not induce persistent effects. The differentiating effects of agents such as Bromo-cAMP and ionomycin are readily reversible, whereas the effects of NGF persist following its removal from the culture medium (Reynolds and Perez-Polo, 1975; Marushige et al. 1987). This concept is so fundamental to the reverse transforming ability of NGF that it requires further emphasis. Because the neoplastic cells remain following reverse transformation, it is essential that the differentiating effects persist. The

aim is to permanently remove neoplastic cells from the replicative-competent pool. Second messenger analogs do not induce persistent effects. It is the persistent nature of the NGF-induced response that makes it particularly attractive as a reverse transforming agent.

Results from in vitro studies (Greene and Tischler, 1976; Reynolds and Perez-Polo, 1981; Sonnenfeld and Ishii, 1982) indicate that NGF is capable of reversing some of the transformed properties of susceptible tumor cells. The data demonstrate that:

1) NGF is capable of initiating a response in a variety of neurogenic tumor cell lines including: pheochromocytoma, glioma and neuroblastoma cell lines;

2) NGF treatment consistently results in the development of a more differentiated phenotype in responsive cell lines;

3) a common consequence of NGF treatment is a reduction in growth rate or complete growth arrest;

4) experiments involving a variety of cell lines indicate that these effects are persistent.

The first attribute indicates that NGF may be effective on a variety of neuroectodermal tumor types. The last three properties were described as crucial attributes of a reverse transforming agent. Together, these results demonstrate that NGF has significant reverse transforming capabilities in vitro.

With this background, let us now examine the results from *in vivo* studies.

In vivo studies

In vitro experiments have contributed a great deal to the wealth of evidence confirming the reverse transforming capabilities of NGF. Such information is an invaluable first step in the process of evaluating the reverse transforming potential of NGF. However, many compounds that prove effective in the petri dish are unable to duplicate this initial promise *in vivo*. This may be due to a variety of factors including an inability to traverse physical and physiologic barriers or a failure to elicit a response in the complex environs of the living organism. Another concern is the potential for systemic toxicity. These considerations necessitate evaluation of the potential therapeutic agent *in vivo*. Upcoming sections address this aspect and provide evidence that NGF also exhibits reverse transforming capabilities *in vivo*.

Models of tumor induction

The first type of *in vivo* system to be discussed will be a model of tumor induction involving the use of ethylnitrosourea (ENU). One of the means by which a treatment can be evaluated is to first induce tumor formation with a chemical carcinogen and then follow with the administration

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of the proposed treatment. Such an approach requires consistent tumor development in a specific organ or tissue. As ENU is capable of inducing a high incidence of neurogenic tumors in susceptible animals, it has proven to be a valuable model for the study of neurogenic tumors. 50 mg/kg ENU administered to pregnant rats on the 20th day of gestation results in the formation of neurogenic tumors in nearly 100% of the offspring (Druckrey et al. 1966a; Ivankovic et al. 1966; Wechsler et al. 1969; Druckrey et al. 1972; Koestner et al. 1971; Swenberg et al. 1972). With such reliable tumor development, this method is an indispensable tool in assessing the effects of therapy on several neurogenic tumor types.

The effect of NGF on the development of ENU-induced tumors has been examined and it appears that NGF levels play a major role in the sensitivity of the nervous system to malignant transformation by ENU. This statement is supported by several lines of evidence:

1) Initial studies showed that treatment of pregnant rats with NGF prior to ENU exposure, or postnatal treatment of the pups, resulted in a significant reduction in early neoplastic proliferations (ENPs) in the trigeminal nerve of the offspring by 90 days of age (Vinores and Koestner, 1982; Camp et al. 1984).

2) In subsequent experiments, treatment of rats with NGF either transplacentally before ENU exposure or postnatally after ENU exposure resulted in a reduction in neoplastic

proliferation in the trigeminal nerves (Vinores and Koestner, 1983).

3) Administration of anti-NGF antibodies resulted in the earlier appearance of tumors suggesting that the level of NGF modulates the carcinogenic response of the nervous system to ENU (Stahn et al. 1975; Vinores and Perez-Polo, 1976).

4) Treatment with NGF resulted not only in a decrease in the number of ENU-induced tumors that developed but also in loss of the neurotrophism that ENU normally exhibits in rats. In other words, when ENU is preceded by NGF, a high incidence of neural tumors is no longer produced by the resorptive carcinogen.

5) Mice, which characteristically have higher physiological levels of NGF than rats, exhibit a neurologically nonspecific response to ENU irrespective of the stage of development and ENU induces very few neural tumors in mice.

6) However, the number of trigeminal nerve neurinomas and central nervous system gliomas in mice is increased nearly fourfold by prior treatment with anti-NGF IgG (Vinores 1976).

The earlier appearance of tumors in groups where anti-NGF antibody treatment preceded ENU exposure suggests that a reduction in the endogenous levels of NGF may prevent the normal NGF-induced maturation of the target cells, thereby increasing the number of ENU-sensitive cells available to the carcinogen. The reduction in neurogenic tumors following

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perinatal NGF administration suggests that NGF may render target cells in the central and peripheral nervous systems more resistant to ENU carcinogenesis, possibly by promoting their maturation.

It does not appear that NGF treatment simply delays the onset of tumor formation. Rather, NGF treatment results in a persistent suppression of tumor development. In a one year study in which the offspring of ENU-treated rats were administered NGF therapy, treatment resulted in a significant ($P < 0.01$) decrease in the number of neurinomas in NGF-treated groups compared to the untreated controls (16 of 34 rats in the NGF treated group developed neurinomas compared to 29 of 34 control animals)(Raju et al. 1989). As the resorptive carcinogen was administered prior to NGF treatment, NGF appears to restrain the promotional phase of tumor development. These results suggest that NGF causes a persistent suppression of the tumor forming ability of ENU-initiated cells.

The nature of the response of ENU-initiated cells to NGF is further clarified by immunohistochemical studies. In untreated animals, 7 of 29 neurinomas exhibited positive immunohistochemical staining for NGF receptors whereas none of the tumors in the NGF-treated group were positive for NGF receptors (Raju et al. 1989). The counterpart to the tumors in the untreated control group that exhibited elevated NGFR was not apparent in the NGF-treated group. Thus, it appears

that NGF treatment suppressed the development of tumors from initiated cells possessing sufficient NGFR to exhibit positive immunohistochemical staining for the receptor protein. These results suggest that NGF treatment restrained the progression of a subpopulation of ENU-initiated cells possessing elevated NGFR.

Results from the *in vivo* models of tumor induction demonstrate three important characteristics of the response to NGF: 1) NGF treatment results in a reduction in the ENU-induced neoplastic proliferation in the trigeminal nerves (Vinores and Koestner, 1983); 2) results from a one year study indicate that these effects appear to be persistent (Raju et al. 1989); and 3) whether or not an initiated cell will respond to NGF treatment appears to be dependent on the presence of NGFR.

Tumor implantation studies

The second system used to evaluate the effects of NGF *in vivo* utilized the implantation of the F98 anaplastic glioma cell line into syngeneic animals. These experiments offer perhaps the most convincing evidence for the *in vivo* efficacy of NGF as they demonstrate that NGF therapy is capable of diminishing the growth of tumor implants in the environs of a living organism. This evidence is supplied by a series of experiments. In the first, rats received intracerebral implants composed of a mixture of F98 anaplastic glioma cells

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and NGF-producing C6 cells. Rats receiving the NGF-producing C6 cells survived longer than those receiving only F98 cells and 30% of these rats were tumor-free at 90 days (Vinores and Koestner, 1981). Additionally, when F98 cells were treated with NGF prior to inoculation, the survival of the recipient was increased significantly ($P < 0.05$) (Vinores and Koestner, 1980). Finally, if untreated F-98 glioma cells were inoculated and the recipient treated with NGF, the animals survived even longer (Vinores and Koestner, 1980). Not only did survival time increase significantly ($P<0.00005$), but also the tumor volume was reduced and the tumor growth rate diminished (Vinores and Koestner, 1980). On histologic examination these tumors exhibited a more differentiated morphology (Vinores and Koestner, 1980).

In vivo tests support in vitro evidence that NGF is capable of reversing some of the transformed properties of neoplastic cells of neurogenic origin and verify that NGF is effective in vivo.

Importance of the NGF-NGFR signal transduction pathway

Before concluding this introductory chapter, it seems appropriate to underscore the relevancy of NGFR expression and the signal transduction pathway. Earlier in this chapter, it was postulated that malignant transformation, or an integral event in malignant transformation, may be associated with an aberrant or incomplete response to normal effectors regulating

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differentiation and growth. A case could be made for this postulate when considering the results from experiments involving several neuroblastoma cell lines. Research has shown that there are at least three different defects in the expression or function of the NGFR in neuroblastoma cell lines, thereby disconnecting one of the normal differentiating pathways in these cells. These defects in the NGF-NGFR pathway may represent an early change in developing sympathetic neuroblasts which leaves them in a proliferative-competent and undifferentiated state in which they may develop subsequent mutations which ultimately lead to malignant transformation (Azar et al. 1990). A proportion of human neuroblastomas have been reported to contain specific abnormalities of chromosome 17 or chromosome 17q (Biedler et al. 1980; Gilbert et al. 1984), which is the chromosome region that carries the NGFR gene in humans (Huebner et al. 1986; Rettig et al. 1986). As malfunction of the NGFR system appears to be relevant in the discussion of neoplastic transformation, activation of the NGF/NGFR system may be relevant in the process of reverse transformation.

Most neuroblastoma cells lack functional NGF-NGFR signal transduction cascade (Matsushima and Bogenmann, 1990). The HTLA NB cell line does not express functional NGFR (Bogenmenn et al. 1983; Matsushima and Bogenmann, 1990). Transfection of the human NGFR cDNA into these cells results in the expression of high-affinity cell membrane-bound NGF receptors

(Matsushima and Bogenmann, 1990). These cells are then able to respond to NGF. They exhibit transient c-fos activation in response to the growth factor which was followed by neurite outgrowth. NGF treatment resulted in the cessation of DNA synthesis as indicated by studies in which 95% of morphologically differentiated cells had not incorporated 3H-thymidine while more than 80% of the non-responsive cells were labeled (Matsushima and Bogenmann, 1990). Differentiated cells maintained their differentiated state upon removal of NGF (Matsushima and Bogenmann, 1990). The authors concluded that, following transfection with NGFR cDNA, NGF was able to stimulate these cells to terminally differentiate (Matsushima and Bogenmann, 1990).

This last section underscores several points that form the basis for our research. It would appear that defects in the NGF-response pathway may be important in neoplastic transformation. Conversely, it is evident that NGF treatment can, in the presence of a functional response pathway, reverse many of the transformed properties of neoplastic cells by inducing a state resembling terminal differentiation.

Summary

Not long after the NGF receptor was discovered, a number of tumor cell lines were identified that possessed abundant NGFR. This finding lead to speculation that aberrant expression of NGF and/or its receptor may have been important

in the process of neoplastic transformation in these cells. That theory has since been discarded. After reviewing the information presented in this chapter, it is easy to understand why this theory was abandoned. In direct contrast to the majority of "growth factors," NGF acts, not as a mitogenic stimulus, but rather, as a differentiating agent. It stimulates both normal and neoplastic cells of neuroectodermal origin to develop the characteristics of more differentiated cells. In the process, it is capable of reversing some of the transformed properties of these neoplastic cells.

The information presented in this introductory chapter leaves little doubt as to the validity and potential benefits of this line of research. These promising results indicate a need to further investigate the reverse transforming potential of NGF. Principal among these needs is the necessity to evaluate additional cell lines for response to NGF in order to develop a better understanding of the spectrum of this compound's potential. As the in vivo effects of NGF have been evaluated in but a single cell line, another research priority is to evaluate additional cell lines in vivo. These considerations will be addressed in upcoming chapters dealing with my thesis research.

CHAPTER 1

THE REVERSE TRANSFORMING EFFECTS OF NERVE GROWTH FACTOR ON FIVE HUMAN NEUROGENIC TUMOR CELL LINES: IN VITRO RESULTS

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CHAPTER 1

The Reverse Transforming Effects of Nerve Growth Factor on Five Human Neurogenic Tumor Cell Lines: *In Vitro* Results.

Abstract

The ability of nerve growth factor (NGF) to stimulate the differentiation of a variety of normal and neoplastic cell lines of neurogenic origin suggests that it has potential as a "reverse transforming agent." The binding of NGF to specific surface receptors may set in motion a series of events whereby neoplastic cells are stimulated to differentiate to a point where they no longer exhibit neoplastic behavior. Such a process is the antithesis of neoplastic transformation and is termed reverse transformation. Five human neurogenic tumor cell lines were evaluated for their response to NGF *in vitro* to examine whether the induced changes were consistent with a reverse transforming response. Crucial alterations essential for reverse transformation include: 1) the development of a more differentiated phenotype, 2) a decrease in the rate of proliferation, and 3) persistence of the induced changes. Each cell line exhibited a morphologic response to NGF which

was generally consistent with the development of a more differentiated phenotype. NGF treatment decreased the growth rate of each cell line and produced complete growth arrest in a single line. The effects of NGF were persistent as the induced morphologic changes remained following removal of NGF from the culture medium. These in vitro experiments indicate that NGF has reverse transforming potential and may prove useful for treatment of neurogenic tumors.

Introduction

The standard approach to cancer therapy generally involves selective removal or destruction of neoplastic cells by surgery, radiation or chemotherapy, or a combination thereof. However, novel therapeutic approaches may be able to augment these traditional treatment strategies. One such option is the management of cancer through control of differentiation and growth of neoplastic cells. Malignant transformation, or an integral event in malignant transformation, may be associated with an aberrant or incomplete response to normal effectors regulating differentiation and growth. If this is true, then specific differentiating agents might be able to stimulate neoplastic cells to differentiate and reverse many of the properties of the transformed cells. Such a process is the antithesis of neoplastic transformation and is termed reverse transformation. It is based on the premise that certain

agents may be able to activate processes which stimulate neoplastic cells to differentiate to a point where they no longer exhibit neoplastic behavior.

Nerve growth factor (NGF) represents one such potential reverse transforming agent. The capability of NGF to stimulate the differentiation of a variety of normal and neoplastic cell lines of neurogenic origin (Waris et al. 1973; Reynolds and Perez-Polo, 1975; Greene and Tischler 1976; Reynolds and Perez-Polo, 1981; Levi-Montalcini and Aloe, 1985; Marushige et al. 1987; Kumar et al. 1990; Matsushima and Bogenmann, 1990) suggests that it may prove useful as a reverse transforming agent. In particular, studies indicating that NGF retards cell proliferation and induces morphologic changes in anaplastic glioma cells (Marushige et al. 1987) and induces the differentiation of PC12 pheochromocytoma (Greene and Tischler, 1976) and neuroblastoma cells (Waris et al. 1973; Reynolds and Perez-Polo, 1981; Perez-Polo et al. 1982; Matsushima and Begenmann, 1990) demonstrate that NGF is capable of reversing some of the transformed properties of neurogenic tumor cells. It is proposed that the differentiating abilities of NGF could be utilized to stimulate neoplastic cells of neurogenic (neuroepithelial) origin to develop a more differentiated phenotype, thereby eliminating the capacity for uncontrolled growth.

The ability to induce a more differentiated phenotype is not unique to NGF. A variety of chemical agents, primarily

analogs of second messengers, also possess this capability (Perez-Polo et al. 1982; Marushige et al. 1989b). However, an important difference exists between these agents and NGF in terms of reverse transforming potential. The differentiating effects of agents such as Bromo-cAMP and ionomycin are readily reversible, whereas the effects of NGF persist following its removal from the culture medium (Reynolds and Perez-Polo, 1975; Marushige et al. 1987). It is the persistent nature of the NGF-induced response that makes NGF particularly attractive as a reverse transforming agent.

The aim of this study was to determine whether NGF was capable of inducing changes indicative of reverse transformation in well characterized cell lines derived from malignant human brain tumors. This was accomplished by evaluating five human neurogenic tumor cells lines for <u>in vitro</u> response to NGF. Each cell line was evaluated for NGF-induced changes in cell morphology. Observations were made as to the capacity of NGF to a) induce morphologic changes suggestive of a more differentiated phenotype and b) decrease the rate of proliferation. As persistence of the induced changes is an essential feature of a reverse transforming agent, an evaluation of how long the NGF induced morphologic changes remained following removal of NGF from the culture medium was also undertaken.

The success or failure of NGF as a treatment depends,

from the onset, on the presence of NGF receptors (NGFR). The initial event in NGF-mediated processes is the binding of the growth factor to specific surface receptors (Levi et al. 1980; Levi-Montalcini 1987; Hempstead et al. 1989). Experimental evidence indicates that the presence or absence of NGFR may determine the outcome of NGF treatment (Raju et al, 1989). Therefore, an essential component of our research was to identify the NGF receptor density of each cell line evaluated.

Investigation of the effects of NGF on growth and differentiation parameters, combined with information on NGFR density, should provide a better understanding of the reverse transforming potential of this growth and maturation factor on human neuroepithelial tumors.

Materials and Methods

Morphology and growth rate studies

Four of the human cell lines used in these experiments, the D54 mixed glioma, glioblastoma multiforme cell lines U118 & U251 and the TE671 medulloblastoma cell line, were generous gifts from Dr. Bigner at Duke University. A fifth cell line, the Hs294 malignant melanoma line, was obtained from the American Type Culture Collection. Stock cultures were maintained in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (Sigma) (10% FBS for the Hs294 cell line) and 4 mM glutamine in a humidified chamber with 8% CO_2

at 37 C. Cultures were seeded in 12 well cluster plates (Corning) at a density of 3.0 x 10^3 to 2.5 x 10^4 cells/well (1.0 ml/well). Cells were seeded in DMEM + 20% FBS. The medium was replaced with HL-1 serum-free medium (Ventrex) after 24 hours. The following day, the medium was again exchanged with HL-1 to which varying concentrations (0.25, 0.5, 1.0, 2.0 and 4.0 ug/ml) of 2.5S NGF (Boehringer Mannheim) were added. Cultures were observed daily via phase-contrast microscopy. For cells that became detached from the culture dish in response to NGF, viability was determined by the trypan blue exclusion test.

Cultures were treated comparably for the growth studies. Paired control and NGF-treated cultures were trypsinized at intervals and counted with either a hemocytometer or Coulter Counter.

Persistence studies

Cultures were treated as for the growth and morphologic studies except seeded at lower densities (5 x 10^3 cells/well). Each 12-well cluster plate was divided into three groups. In the first, the NGF-control group, the medium was refreshed with 4 ug/ml NGF in HL-1 every fourth day throughout the experiment. In the second, the persistence group, cells were cultured in the presence of NGF for 48 hrs. The NGF-containing medium was then removed and replaced with HL-1. In the third group, the untreated control group, cells were

grown in HL-1 throughout the experiment. The culture medium was refreshed with HL-1 every 4th day in both the HL-1 control and NGF-persistence cultures. Cultures were observed daily via phase-contrast microscopy.

NGF receptor studies

NGF receptor (NGFR) density was determined for each cell line using a modification of the ELISA procedure described by Doherty et. al. (1988). Cells were seeded at 15,000 cells/well in a 96-well microtiter plate. Following two days in culture, the cells were fixed with paraformaldehyde. Receptor density was determined using the ME20.4 anti-receptor antibody (1:20, Amersham) and an anti-mouse horseradish peroxidase conjugate (1:1000, Sigma). Optical density (OD) was measured with the Dynatech MR600 microtiter plate reader. A standard curve was developed using a cell line with known NGF-binding properties, the Hs294 malignant melanoma cell line (Fabricant et al. 1977). OD was determined at various Hs294 seeding densities. Protein concentrations were determined from which cell numbers were calculated. A standard curve was generated that compared OD to Hs294 cell numbers. Unknowns were then compared to the standard curve.

Effect of preincubation with ME20.4

ME20.4 is a monoclonal antibody that binds to the human NGFR and in so doing inhibits the binding of NGF to its

receptor (Ross et al. 1984). Preincubation of cultures with ME20.4 should greatly diminish or prevent the development of NGF-induced changes. Cell lines were seeded at 1.25 x 10^4 cells/well in a 12-well cluster plate. On the second day the medium was replaced with HL-1. On the third day, the plate was divided into 6 groups; an HL-1 control group, two NGF-treated groups (2.0 and 4.0 ug/ml NGF), an ME20.4 control group (1.8 ug/ml) and two groups which were pre-incubated with ME20.4 (1.8 ug/ml) 60 min. prior to the addition of either 2.0 or 4.0 ug/ml NGF. Groups were compared to determine whether pre-incubation with ME20.4 could prevent or diminish the NGF-induced morphologic changes.

Results

An ELISA procedure was developed to determine NGFR density (Table 1.1). Results from these tests indicated that the Hs294 cell line had abundant NGFR. Tumor cell lines of glial origin had very few NGFR. The TE671 medulloblastoma cell line exhibited a slightly higher density of receptors than the glial tumor lines. The relative densities obtained were consistent with those reported for cell lines of similar origin (Fabricant et al. 1977). Receptor numbers were estimated using information from published reports (Fabricant et al. 1977). These calculations indicate that the Hs294 cell line would have approximately 6.4 x 10^5 receptors per cell, the glial cultures approximately 6.0 - 10.0 x 10^3

Table 1.1. Relative NGFR densities of the five human neurogenic tumor cell lines

Cell Line	Binding of ME20.4 ([a]Arbitrary units ± S.E.)
Hs294	106.7 ± 12.36
TE671	2.9 ± 0.58
D54	1.7 ± 0.34
U251	1.4 ± 0.17
U118	1.0 ± 0.31

Table 1.1 illustrates the relative NGFR density of the human tumor cell lines expressed in terms of the binding of ME20.4 (antibody directed against the human NGFR) as determined by standard ELISA.

[a] 1.0 arbitrary unit corresponds to 0.165 O.D. units per 2.5×10^4 cells.

receptors/cell and the TE671 cell line approximately 1.7 x 10^4 receptors/cell.

NGF treatment resulted in the morphologic transformation of each of the five human neural crest tumor cell lines evaluated. The type of response and dose at which it was observed varied with the cell line (Table 1.2). In general, a morphologic response was first identified at a dose between 0.25 and 0.5 ug/ml NGF and was maximal at concentrations of 2.0 to 4.0 ug/ml NGF. Two of the cell lines, D54 and U118, responded in a similar manner. Within 8 hours following exposure, cells became more compact, 3-dimensional and produced an increased number of very fine, short processes. Over the next 16 to 24 hours, the cells became increasingly more compact, eventually attaining a spherical shape with concomitant loss of their processes. After 24 to 48 hours, most of the cells had detached from the surface of the culture dish and died. Cells that remained attached tended to be present in clusters exhibiting numerous short, fine peripheral processes. (Figure 1.1 C-D)

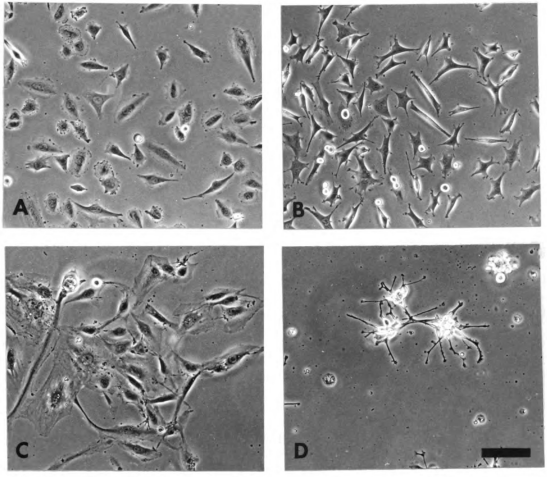
The remaining three cell lines (Hs291, U251 and TE671) exhibited a similar initial response. Cells became more compact, 3-dimensional and produced more highly developed processes. However, rather than becoming spherical and detaching from the culture dish, these cells developed a more differentiated phenotype characterized by an increasingly more compact and 3-dimensional morphology and the production of

Table 1.2: Time and dose rate parameters for the response of the human tumor cell lines to NGF <u>in vitro</u>.

Cell line:	U251	Hs294	D54	TE671	U118
Earliest observed mophologic changes at 4.0 ug/ml NGF:	24 hrs	24 hrs	6 hrs	24 hrs	8 hrs
Minimum NGF dose (ug/ml) required for morphologic response:	0.5	0.25	0.25	0.5	0.25
Minimum NGF dose (ug/ml) required for maximal response:	4.0	2.0	4.0	4.0	4.0

Table 1.2 is an outline of the basic response parameters for each of the five human neurogenic tumor cell lines. The table indicates the earliest time at which a morphologic change was observed following maximal stimulation; the minimal NGF concentration (ug/ml) required to achieve an identifiable morphologic change, and the concentration of NGF (ug/ml) at which a maximal response was elicited.

Figure 1.1: Phase-contrast photomicrographs of the NGF-induced morphologic changes in the D54 (mixed glioma) and Hs294 (malignant melanoma) cell lines.

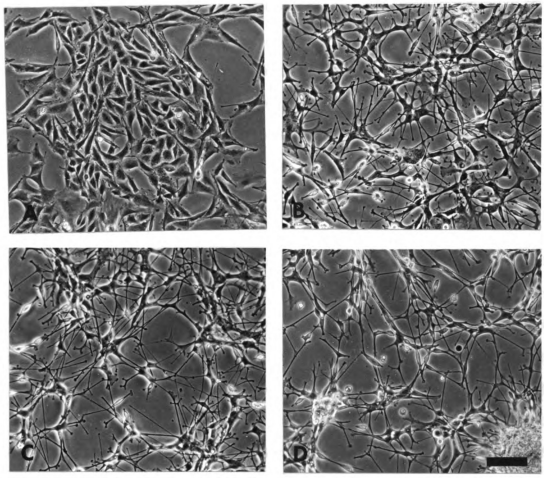


Phase-contrast photomicrographs of the following groups were taken on day 4 post-NGF treatment: A) Hs294, untreated control; B) Hs294, 4 ug/ml NGF; C) D54, untreated control; D) D54, 2 ug/ml NGF. In response to NGF the Hs294 cell line develops a more compact, three-dimensional morphology and numerous short processes. The D54 cell line responds to NGF by becoming considerably more compact and by developing multiple, long, slender processes. (Bar equals 10 um)

numerous, well developed processes. This change varied from the production of numerous short processes (Figure 1.1 A-B) to the development of an extensive network of long slender branching processes that formed an interconnecting network with neighboring cells. (Figure 1.2 A-B) These changes were quite stable in the U251 cell line. However, following the development of a maximal morphologic response, cells from both the Hs294 and TE671 lines would gradually begin to detach from the culture dish.

Two procedures were undertaken to try and determine if the morphologic changes were the result of binding of NGF to specific surface receptors. In the first, 2.0 ug/ml cytochrome C, a compound similar to NGF in both size and charge (Korshing and Thoenen, 1983), was added to the culture medium to determine if the NGF-induced morphologic changes were caused simply by the addition of a positively charged molecule to the culture medium. Differences between the cytochrome C-containing cultures and untreated control cultures were not identified, indicating that the changes observed following NGF treatment were apparently not due to the addition of a charged molecule. Preincubation with ME20.4, an antibody that binds specifically to the human NGFR (Ross et al. 1984), greatly diminished the development of NGF induced morphologic changes, suggesting that the morphologic transformation induced by NGF was the result of the binding of the growth factor to specific surface receptors.

Figure 1.2: Phase-contrast photomicrographs of the NGF-induced morphologic changes in the U251 glioblastoma multiforme cell line.

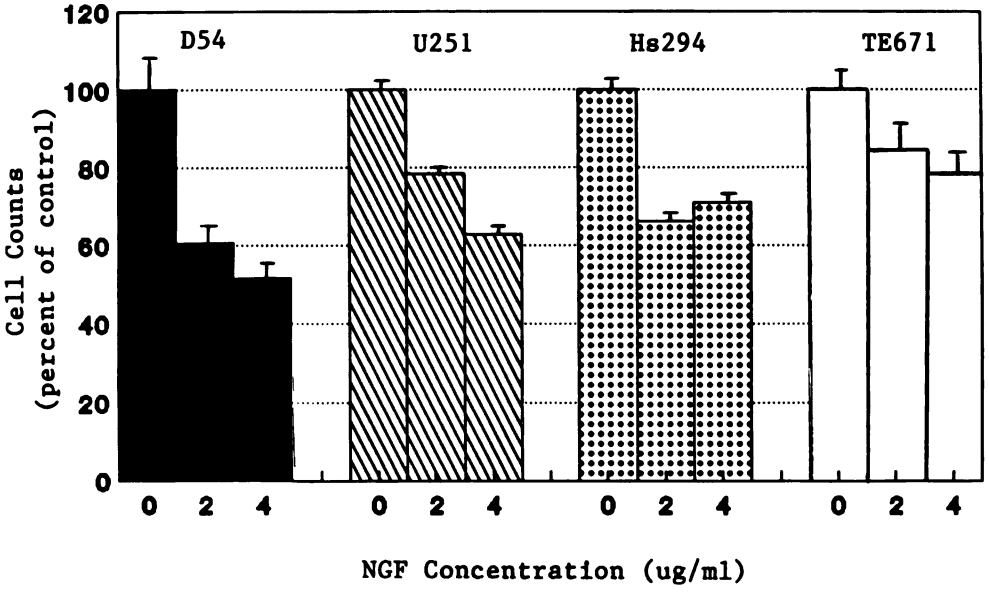


Phase contrast photomicrographs of the following groups were taken: A) U251, untreated control, day 11; B) U251, 4 ug/ml NGF, day 11; C) U251-Persistence, NGF control, 4 ug/ml NGF every fourth day, day 43; D) U251-Persistence, persistence group, 4 ug/ml NGF day 1-2, HL-1 media change every fourth day, day 43. In response to NGF, the U251 cell line becomes much more compact, three-dimensional and develops an extensive network of long slender branching processes that tend to form an interconnecting network with neighboring cells. These changes are quite persistent as is evidenced by the retention of the NGF-induced changes in morphology even after the cells had been cultured in the absence of NGF for 41 days. (Bar equals 10 um)

The morphologic changes induced by NGF were accompanied by a decrease in growth rate in each cell line. On day 4 post-NGF treatment, this ranged from a minimal decrease in the TE671 cell line, to a nearly 50% decrease in the D54 cell line (Figure 1.3). Due to various technical problems (cells becoming detached in response to NGF), the development of a complete growth response curve was possible only in the U251 cell line. NGF treatment resulted in a reduction in the growth rate of this cell line with complete cessation of growth after day 10-12 (Figure 1.4).

An evaluation of how long the NGF-induced morphologic changes persisted in the absence of the compound was also undertaken. Persistence was documented in the D54, Hs294 and U251 cell lines. The evaluation was limited by the varying behavior and survival of the cells in culture. In the D54 cell line, the NGF-induced morphologic changes persisted for 7 days following removal of NGF from the culture medium. Changes persisted in the Hs294 cell line for 14 days following removal of NGF. These values were not a true reflection of the persistent nature of the NGF-induced response in these cell lines. Even when grown on polylysine coated plates, cells from D54 cultures became detached in response to NGF after 7 days. In the Hs294 cell line, morphologic changes became obscured once these cultures became confluent at approximately day 14. At the termination of each experiment, there was no evidence of reversal of the NGF-induced

Figure 1.3: The effect of NGF on the cell counts of four human neurogenic tumor cell lines on day 4 post-NGF treatment.



Cell Counts, Day 4 post-NGF treatment. Values are expressed as a percentage of the corresponding control (± S.E.) so that the cell lines can be readily compared.

Figure 1.4: The effect of NGF-Treatment on the growth rate of the U251 Glioblastoma multiforme cell line *in vitro*.

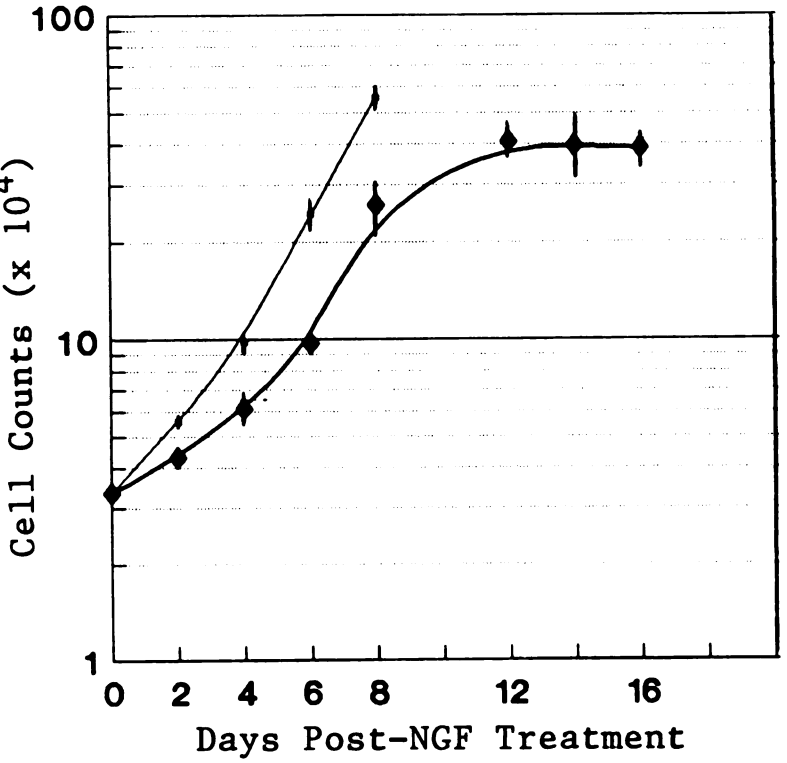


Figure 1.4 illustrates the effect of NGF-treatment on the growth curve of the U251 glioblastoma multiforme cell line. Untreated control cultures (•) rapidly proliferate and reach confluency by day 8 whereas the 4.0 ug/ml NGF-treated (•) cultures exhibit a gradual reduction in growth rate with eventual growth arrest by day 10.

morphologic changes. Thus, it was unclear how much longer the effects would have persisted had technical problems not prohibited further evaluation. Persistence was best demonstrated in the U251 cell line (Figure 1.2 C-D) where the NGF-induced morphologic changes persisted for 53 days following removal of NGF from the culture medium. Cells which were exposed to NGF for 2 days retained a morphology essentially identical to that of cells in NGF control cultures and showed no evidence of reverting to a morphology similar to untreated controls even after they had been grown without NGF for 53 days. The experiment was terminated at this point due to the unhealthy condition of the cultures following prolonged maintenance in HL-1.

Discussion

Control of growth and differentiation of tumor cells is a basis for cancer therapy and is the principle behind the concept of reverse transformation. However, since neoplastic cells are not directly eliminated through the use of reverse transforming agents, very stringent criteria need to be met when evaluating such compounds as potential therapeutic agents. Treatment must render neoplastic cells susceptible to the body's intrinsic control mechanisms or result in a persistent suppression of growth if a therapeutic advantage is to be gained.

Our data indicate that NGF is able to induce several

changes in neoplastic cell lines critical for reverse transformation. It was capable of inducing a more differentiated phenotype and of reducing or arresting tumor growth *in vitro*. These are both vital components of a reverse transforming response. However, in order for a reverse transforming agent to be therapeutically useful, an additional characteristic is desired. An valuable feature of agents that rely on differentiation as a means of producing a therapeutic effect is persistence of the differentiated phenotype. NGF induced changes were highly persistent *in vitro*. It is the persistent nature of this response that separates NGF from many other compounds capable of inducing differentiation and suggests that NGF may prove particularly useful as a reverse transforming agent. Furthermore, reports indicate that the effects of NGF may also be persistent *in vivo*. Following a one year study in which neurogenic tumors were induced by transplacental exposure to ethylnitrosourea (ENU), significantly fewer animals developed neurinomas in the NGF-treated group (47% as compared to 85% for the untreated controls; $P < 0.01$), indicating that NGF has the ability to persistently suppress the tumor forming ability of ENU initiated cells (Raju et al. 1989). As the resorptive carcinogen was administered prior to NGF treatment, NGF appears to restrain the promotional phase of tumor development.

It is generally believed that NGF-responsive cells

possess two types of NGFR, high affinity receptors (thought to be necessary for biologic response) and low affinity receptors (Landreth and Shooter, 1980; Sonnenfeld and Ishii, 1985; Vale and Shooter, 1985; Green and Greene, 1986; Kumar et al. 1990; Welcher et al. 1991). However, a recent report (Buxser et al. 1990) provides strong evidence for the existence of but a single type of NGFR. The confusion appears to stem from the internalization of the NGF-NGFR, mimicking a second class of receptors (Buxser et al. 1990). In our experiments, an ELISA was used to determine the NGFR-densities of the various cell lines under study (Table 1.1). The relative densities obtained were consistent with information on similar cell lines (Fabricant et al. 1977). A correlation between receptor density and response to NGF was not identified, suggesting that additional factors are involved in the responsiveness of cell lines to NGF besides NGFR density. The Hs294 cell line, which possessed the highest density of NGFR, exhibited a maximal response at the lowest dose of NGF, however, the magnitude of this response was not as great as was observed in several cell lines exhibiting fewer receptors. The cell line of intermediate density, the TE671 line, proved to be the least responsive cell line. Three of the cell lines, D54, U251, U118, had low NGFR levels. Despite low receptor numbers, a reverse transforming response was elicited in each cell line, albeit at high doses of NGF. These results indicate that low levels of NGFR, such as were

identified in the cell lines of glial origin, were not a significant obstacle to reverse transformation if ample NGF was delivered to the neoplastic cells. Stereotaxic treatment, which enables the targeting of agents to specific sites, may allow for the administration of a sufficient dose of NGF to neoplastic foci to stimulate a reverse transforming response in vivo.

The mechanisms by which NGF affects normal and neoplastic cells are currently not completely understood. However, if NGF is to be useful as a reverse transforming agent, it must have persistent epigenetic effects that reverse or block one or more of the steps that lead to neoplastic transformation. It has been shown that NGF treatment results in the heterologous down-regulation of epidermal growth factor receptors (Lazarovici et al. 1987). Thus, as part of its differentiating response, NGF appears to diminish pathways that favor cell proliferation. This may be a part of the mechanism whereby the differentiated phenotype is maintained (Lazarovici et al. 1987).

In vitro tests indicate that NGF fulfills several key criteria of a reverse transforming agent. NGF treatment results in a reduction in growth rate with complete growth arrest in some cell lines. It stimulates neoplastic cells to develop a more differentiated phenotype and these effects appear to be highly persistent. As such, it appears that NGF possesses significant therapeutic potential. Work is

currently underway to characterize the reverse transforming potential of NGF *in vivo*.

CHAPTER 1 APPENDIX

Introduction

The preceding paper provides information supporting the reverse transforming potential of NGF *in vitro*. The material presented did not include all the information obtained from *in vitro* studies. The scope of the article was limited to information necessary to demonstrate the reverse transforming capabilities of NGF in a tissue culture system. Additional *in vitro* studies were performed. The results of these experiments are reported here, in the appendix, because they provide additional information on the response of these neoplastic cell lines to NGF in tissue culture.

NGF-induced morphologic changes

The preceding article did not allow for sufficient space to illustrate the spectrum of morphologic changes induced by NGF in all five of the neoplastic cells lines under study. This appendix provides the opportunity to illustrate these changes and to furnish a visual record of the NGF-induced changes in morphology. The "Materials and Methods" for this sections are described in the preceding article.

U118 Glioblastoma Multiforme

The U118 glioblastoma multiforme cell line exhibits an orderly progression of changes in response to NGF. The initial NGF-induced changes consist of the cell bodies becoming shortened and more compact with loss of cytoplasmic processes (Figure 1.5). As these changes progress, cells become even more compact, three-dimensional and eventually develop a spherical shape. These spherical cells then detach from the surface of the culture dish. This progression can be observed either by examining a single treatment group over a period of several days or by examining the various NGF treatment groups from a single time period. The first changes were noted in the 4 ug/ml group following 8 hrs' exposure to NGF. Morphologic changes were observed at a dose as low as 0.25 ug/ml NGF and were maximal at 4.0 ug/ml NGF. The NGF-induced morphologic changes reached their peak by day 4 post-NGF treatment.

Figure 1.5: Phase-contrast photomicrographs of the spectrum of morphologic changes induced by NGF in the U118 glioblastoma multiforme cell line.

Photomicrographs were taken at phase 1. (Bar = 10 um)

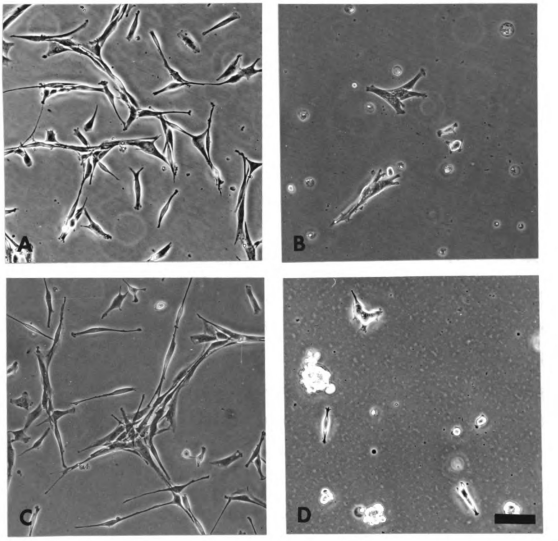
A: Day 1 post-NGF treatment, HL-1 control: Control cultures consist of a population of large, fairly flat spindle shaped cells that generally possess from 1 - 2 processes per cell. Occasional cells exhibit an angular or stellate morphology.

B: Day 1 post-NGF treatment, 4 ug/ml NGF: Approximately 30% of the cells in this group have become detached from the surface of the culture dish. The majority of the cells that remain are spherical in shape. The few non-spherical cells present are greatly shrunken and are devoid of processes.

C: Day 3 post-NGF treatment, HL-1 control: Cells in the control cultures have retained a morphology similar to that described for the day 1 control cultures.

D: Day 3 post-NGF treatment, 4 ug/ml NGF: The majority of cells in this culture have become spherical and detached from the culture surface. Compared to the control cultures, there are approximately 70% fewer cells. 80-90% of the cells that remain attached are spherical. A few of the cells that remain are markedly shrunken, rod shaped and devoid of processes.

Figure 1.5

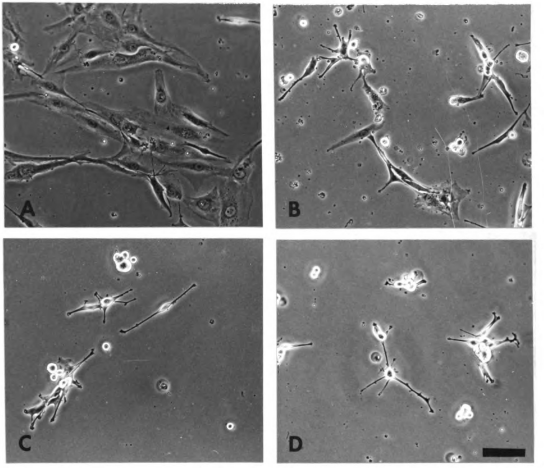


D54 Mixed Glioma

Components of the NGF-induced morphologic changes exhibited by the D54 mixed glioma cell line are reminiscent of changes observed with the U118 cell line. This cell line also tends to become spherical and detach from the surface of the culture dish in response to NGF. However, before this stage is reached, these cells undergo a more dramatic spectrum of morphologic changes than is observed with U118 cultures. Whereas the U118 cell line tends to become progressively more compact until it eventually reaches a spherical shape, the D54 cell line progresses through a stage where cells exhibit well developed processes.

Untreated D54 cultures consist of a population of very large, flat, two-dimensional, irregular to spindle shaped cells. NGF exposure stimulates these cells to become more compact and three-dimensional. Cells then become compressed into long, slender, three-dimensional cells that often exhibit well developed processes at one or both poles (Figure 1.6). These cells progress to become considerably more compact and develop a number of long slender processes. The processes eventually condense and the cells become spherical and detach from the culture surface. In response to NGF the D54 cell line exhibits the most dramatic decrease in size of any of the five human cell lines evaluated. These cells progress from very large flat cells to cells with minute cell bodies possessing long, slender processes.

Figure 1.6: Phase-contrast photomicrographs of the spectrum of morphologic changes induced by NGF in the D54 mixed glioma cell line.



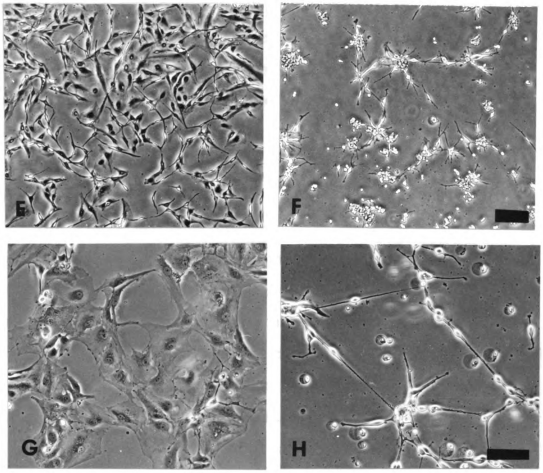
Photomicrographs were taken at phase 1 on day 3 post-NGF treatment. (Bar = 10 um)

A: HL-1 control: cells in the control cultures consist of a population of very large, flat, fusiform to irregular cells that occasionally exhibit short processes.

B: 1.0 ug/ml NGF: These cells differ from the controls in that they have become much more compact and three-dimensional, and are beginning to sprout an increased number of short processes.

C: 2.0 ug/ml NGF: Compared to untreated control cultures, the cell density in this group has become greatly decreased as many of the cells have become spherical and have detached. Those that remain are even more compact and exhibit long, slender processes.

D: 4.0 ug/ml NGF: The cell density in this group has decreased compared to the previous group as cells continue to detach. Those that remain are many times smaller than the untreated controls and exhibit well developed processes.



1.6E: Day 1, HL-1 control and F: Day 1, 4.0 ug/ml NGF: These two photomicrographs were taken at phase L (Bar = 20 um) to better demonstrate the proportion of cells that become spherical and detach in response to NGF and how NGF treatment stimulates these cells to become significantly more compact. Fewer cells are present in the 4.0 ug/ml NGF-treated group. The cells that remain have not retained the flat, spindle-shaped morphology of cells in the corresponding control cultures. These cells have become significantly more compact. They will eventually attain a spherical shape and detach.

1.6G: Day 9, HL-1 control, and H: Day 9, 0.8 ug/ml NGF, phase 1 (Bar = 10 um): A fairly low dose of NGF was utilized so that the morphologic changes would become more developed before the cells detached. These two figures illustrates how compact and three-dimensional the cells become in response to NGF and how NGF will induce these cells to develop long slender processes that from an interconnecting network with neighboring cells.

In the D54 cell line NGF-induced changes in morphology were noted in the 4 ug/ml group following 6 hrs' exposure to NGF. Morphologic changes were observed at a dose as low as 0.25 ug/ml NGF and were maximal at 4.0 ug/ml NGF. The NGF-induced morphologic changes reached their peak by day 4 post-NGF treatment.

TE671 Medulloblastoma

The TE671 medulloblastoma cell line undergoes possibly the most subtle range of morphologic changes in response to NGF. Though these changes are not as dramatic as the other four cell lines, they are readily apparent and are consistent with the development of a more differentiated phenotype. This cell line develops an orderly progression of morphologic changes in response to NGF. This progression can be illustrated by comparing the various NGF treatment groups on day 3 post-NGF treatment (Figure 1.7). Untreated TE671 cultures consist of a population of very large, flat, two-dimensional, spindle-shaped cells that only occasionally exhibit well developed processes. Low levels of NGF (0.25 and 0.5 ug/ml) stimulate these cells to develop a more compact, oval to angular, more three-dimensional morphology with an increased number of cells exhibiting short processes. As the dose of NGF increases, the cells become progressively more compact and three-dimensional, a greater number of cells produce well developed processes and these processes tend to

Figure 1.7: Phase-contrast photomicrographs of the spectrum of morphologic changes induced by NGF in the TE671 Medulloblastoma cell line.

Photomicrographs were taken at phase 2 on day 3 post-NGF treatment. (Bar = 5 um)

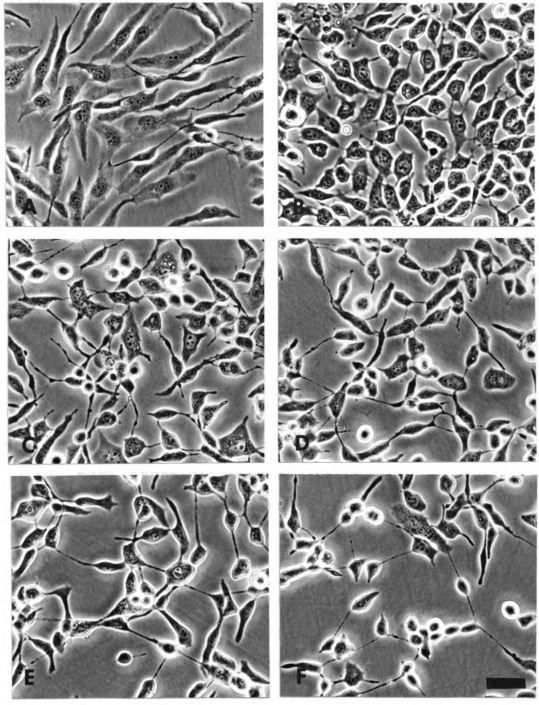
A: HL-1 control: Control cultures consist of a population of large, flat, two-dimensional, spindle shaped cells that exhibited an occasional well developed polar process.

B: 0.25 ug/ml NGF: When compared to the control cultures, cells in this group are more compact, slightly more three-dimensional and have developed an oval to angular morphology.

C: 0.5 ug/ml NGF: Cells in this group have become even more compact and three-dimensional than the previous group and are beginning to exhibit an increased number of moderately well developed processes.

D:1.0 ug/ml, E: 2.0 ug/ml and F: 4.0 ug/ml: As the dose of NGF increases to 1.0 ug/ml and above, cells continue to become progressively more compact, three-dimensional and produce and increased number of well developed processes that tend to form an interconnecting network with neighboring cells.

Figure 1.7



form an interconnecting network with neighboring cells.

The earliest identifiable morphologic changes were apparent following 24 hrs exposure to 4.0 ug/ml NGF. Morphologic changes were observed at doses as low as 0.25 ug/ml NGF and were maximal at 4.0 ug/ml NGF. The NGF-induced morphologic changes reached their peak by day 4 post-NGF treatment.

Hs294 Malignant Melanoma

The Hs294 malignant melanoma cell line undergoes an interesting succession of morphologic changes. The predominant cell type in untreated Hs294 cultures is a very primitive appearing large, flat, two-dimensional, amorphous cell that is devoid of processes. In response to NGF these amorphous cells begin to develop a more compact, angular, three-dimensional morphology. Whereas the amorphous cells could be considered the most undifferentiated cell type, these angular, more three-dimensional cells are an intermediate stage in the progression towards the development of a more differentiated phenotype. As either the length of exposure or the dose of NGF increases, these angular cells become more compact, three-dimensional and begin to sprout processes. These cells eventually develop a stellate morphology and produce numerous, short, often branching processes in response to NGF. These stellate cells represent the most differentiated state that Hs294 cells develop in response to

NGF. Though these NGF-induced changes may not be as dramatic as those exhibited by the D54 and U251 cell lines, they are readily apparent and the transition from a population of primitive amorphous cells to highly differentiated three-dimensional cells exhibiting numerous processes effectively demonstrates the ability of NGF to induce Hs294 malignant melanoma cells to develop a more differentiated phenotype.

The first NGF-induced morphologic changes were noted in the 4 ug/ml group following 24 hrs' exposure to NGF. Morphologic changes were observed at a dose as low as 0.25 ug/ml NGF and were maximal at 2.0 ug/ml NGF. The NGF-induced morphologic changes reached their peak by day 5 post-NGF treatment.

Figure 1.8: Phase-contrast photomicrographs of the spectrum of morphologic changes induced by NGF in the Hs294 Malignant Melanoma cell line.

These photomicrographs were taken at phase 1 on day 2 post-NGF treatment. (Bar = 10 um)

A: HL-1 control: The predominant cell type in the control cultures was a population of very large, flat, two-dimensional, amorphous cells that were essentially devoid of processes. A few scattered spindle shaped cells, angular cells and an occasional stellate cell are also apparent.

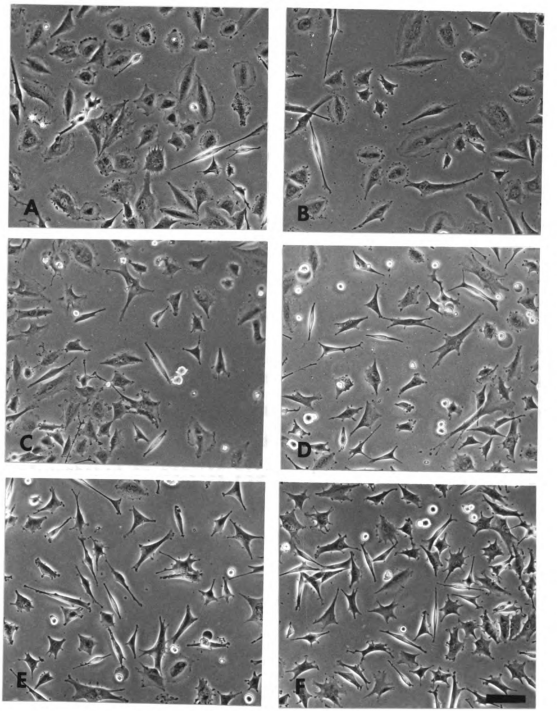
B: 0.25 ug/ml NGF: Compared to the control cultures, cells in the 0.25 ug/ml NGF-treated group exhibited a decreased number of amorphous cells with a corresponding increased number of angular, more three-dimensional cells exhibiting short processes.

C: 0.5 ug/ml NGF: The morphologic changes are more progressed in this group. The percentage of amorphous cells has gradually decreased compared to the two previous groups while the number of angular and stellate cells continues to increase.

D: 1.0 ug/ml NGF: Classical amorphous cells are rarely identified in this group or in either of the two groups where higher doses of NGF were employed. Cells in this group consistently exhibit a more three-dimensional morphology and the majority have sprouted short processes.

E & F: 2.0 & 4.0 ug/ml NGF: The morphologic changes present in these two groups are similar to those described in the previous group, however, changes continue to progress. Cells in these two groups are even more compact, three-dimensional and exhibit increased numbers of processes.

Figure 1.8



U251 Glioblastoma Multiforme

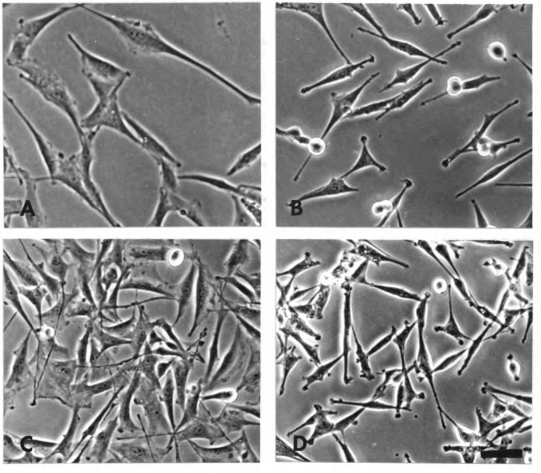
The U251 glioblastoma multiforme cell line exhibits perhaps the most pronounced morphologic changes in response to NGF, however, it takes significantly longer for these morphologic changes to become fully developed. Whereas the other four cell lines exhibit fully developed morphologic changes following 3-4 days' exposure to NGF, it takes 11-12 days for morphologic changes to become fully developed in the U251 cell line. These cells eventually develop striking morphologic changes that, once again, readily demonstrate the ability of NGF to stimulate neoplastic cell lines of neurogenic origin to development morphologic characteristics of a more differentiated phenotype.

The morphologic changes in the U251 cell line are best followed by examining the changes in the 4.0 ug/ml NGF-treated group over time. Initially, U251 control cultures consist of a population of very large, flat, two-dimensional, spindle-shaped to angular cells. Following two days exposure to NGF, these cells become much more compact, three-dimensional and a larger proportion of cells attain a spindle-shaped appearance. As the cell density increases in the control cultures, the cells become increasingly flattened, angular and generally exhibit a single process or lack processes altogether. NGF-treated cultures exhibit a dramatically different morphology. The spindle-shaped cells in NGF-treated cultures become much more compact and three-

dimensional. As the length of exposure to NGF increases, these cells develop processes which gradually lengthen and branch until, by day 11-12, NGF-treated cultures consist of a population of cells with very compact, three-dimensional cell bodies that have developed numerous long, slender, often branching processes that tend to form an interconnecting network with neighboring cells.

The earliest observed morphologic changes were noted in the 4 ug/ml group following 24 hrs' exposure to NGF. Morphologic changes were observed at a dose as low as 0.5 ug/ml NGF and were maximal at 4.0 ug/ml NGF.

Figure 1.9: Phase-contrast photomicrographs of the spectrum of morphologic changes induced by NGF in the U251 glioblastoma multiforme cell line.



Photomicrographs were taken at phase 2. (Bar = 5 um)
Day 2 Post-NGF treatment:

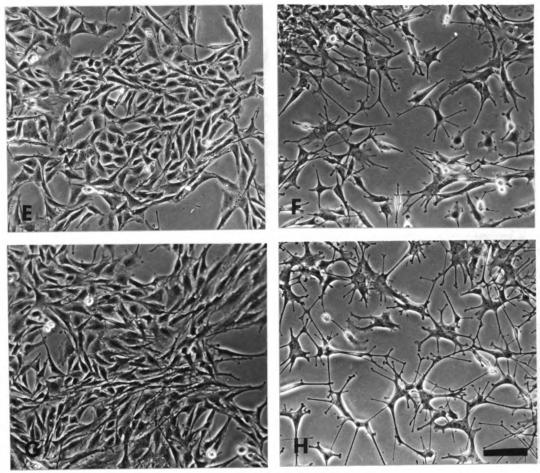
A: HL-1 control: Control cultures consist of a population of very large, flat cells that exhibit an irregular to spindle-shaped morphology.

B: 4.0 ug/ml NGF: Cells in the 4.0 ug/ml NGF-treated cultures exhibit a significantly more compact, three-dimensional morphology than cells in the untreated control cultures.

Day 6 Post-NGF treatment:

C: HL-1 control: Cells in the control cultures are very flattened, angular and exhibit 0-1 processes per cell.

D: 4.0 ug/ml NGF:. Cells in the 4.0 ug/ml NGF-treated cultures are much more compact, three-dimensional and tend exhibit a spindle-shaped to stellate morphology.



Photomicrographs were taken at phase 1. (Bar = 10 um)
Day 9 Post-NGF treatment:

1.9E: HL-1 control: Cells in the control cultures remain flat and angular and exhibit few processes.

1.9F: 4.0 ug/ml NGF: Cells in the 4.0 ug/ml NGF-treated cultures are more compact, three-dimensional and are beginning to develop numerous processes.

Day 11 Post-NGF treatment:

1.9G: HL-1 control: On day 11 post-NGF treatment, cells in the control cultures continue to exhibit a flat, angular morphology and exhibit few processes.

1.9H: 4.0 ug/ml NGF: 4.0 ug/ml NGF-treated cultures consist of a population of cells that have very compact, three-dimensional cell bodies and have developed numerous long, slender, often branching processes that tend to form an interconnecting network with neighboring cells.

Discussion

Descriptions of the NGF-induced morphologic changes contain a recurring theme. In response to NGF, each of the tumor cell lines become more compact and three-dimensional, and in four of the five cell lines NGF stimulates cells to produce well developed processes. These types of changes are significant because the induction of a more compact, three-dimensional morphology and the production of well developed processes are suggestive of the development of a more differentiated phenotype. One of the goals of these *in vitro* experiments was to demonstrate that NGF was capable of inducing the development of a more differentiated phenotype. Information presented in the preceding paper and in this appendix provides ample evidence that NGF is able to induce the five neurogenic tumor cell lines under study to develop morphologic characteristics of a more differentiated phenotype. These NGF-induced morphologic changes are readily visualized. Some of the information provided in these photomicrographs is also amenable to image analysis. The succeeding section on image analysis provides additional support for the ability of NGF to induce dramatic changes in cell morphology that are consistent with the development of a more differentiated phenotype.

Image Analysis

The preceding article states that the neurogenic tumor cell lines evaluated in these studies typically respond to NGF by becoming more compact, three-dimensional, and by producing well developed processes. These types of changes are indicative of the development of a more differentiated phenotype. Though these changes are readily visualized, they are difficult to quantify. In particular, precise measurement of three-dimensional changes are difficult to obtain. However, measurement of alterations in maximal cross-sectional area, which accompany changes in three-dimensional morphology, and changes in the number of well developed processes are readily obtained. These two parameters were measured in order to collect quantitative information on NGF-induced changes in morphology.

Materials and Methods

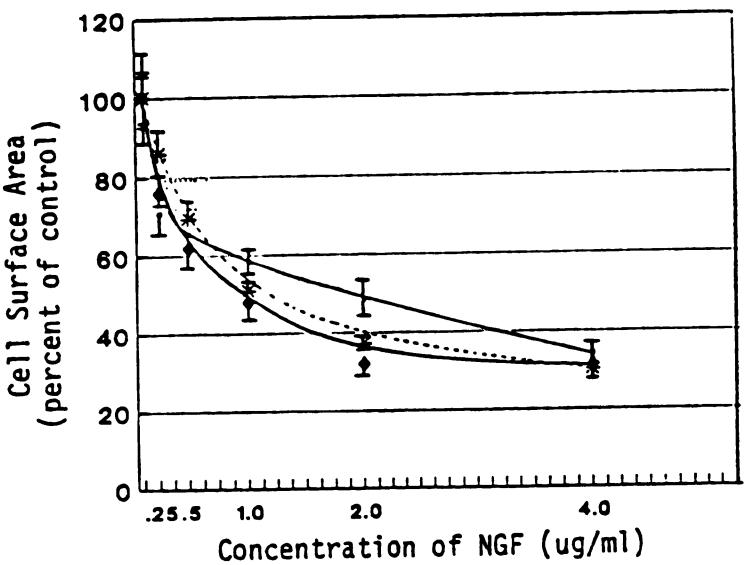
The Hs294 malignant melanoma, TE671 medulloblastoma and U251 glioblastoma multiforme cultures were evaluated in this study. Phase contrast photomicrographs of cultures described in the preceding paper under "Morphologic Studies" were analyzed with the Sigma Scan (Jandel Scientific) program. The dose of NGF and length of NGF exposure of the analyzed cultures are described in the Results section. Analysis was performed on Phase 2 photographs of the U251 and TE671 cell lines and on Phase 1 photographs of the Hs294 cell line. The

criteria for counting a process varied with the cell type. U251 and TE671 cells developed long, slender processes in response to NGF. For these two cell lines, the process had to be at least twice the length of the average cell body in order to be counted. In contrast to the previous two cell lines, the cell bodies of the Hs294 cell line were larger and the processes considerably shorter. In order for a process to be counted in this cell line, the process had to be at least 1/2 the length of the average cell body.

Results

Image analysis was not performed on the D54 and U118 cell lines because they readily became detached in response to NGF. Image analysis of the U251, TE671 and Hs294 cell lines was performed. Figure 1.10 illustrates the dramatic effect that increasing doses of NGF had on the maximal cross-sectional area of these cells. Increasing concentrations of NGF resulted in a dramatic decrease in cross-sectional area. At 4 ug/ml NGF, the maximum concentration of NGF utilized in these studies, the average cross-sectional area of these neoplastic cell lines was only 30.3 - 34.1% that of the untreated control cultures. The U251 cell line exhibited a dramatic decrease in cross-sectional area on day 6 post-NGF administration, the measurement period in which image analysis was performed. However, morphologic changes in these cells continued to develop after this time. The cells became even

Figure 1.10: The effect of NGF treatment on maximal cross-sectional area of the TE671, U251 and Hs294 cell lines.



Maximal cross-sectional area measurements of the TE671 (medulloblastoma), U251 (glioblastoma multiforme), and Hs294 (malignant melanoma) cell lines were generated from photomicrographs taken of cultures on days 3, 6 and 13 respectively, post-NGF treatment. Measurements were taken at five different NGF concentrations (0.25, 0.5, 1.0, 2.0, and 4.0 ug/ml NGF) for each cell line. Increasing doses of NGF result in a corresponding decrease in maximal cross-sectional area.
(•) Hs294
(+) TE671
(•) U251

more compact, three-dimensional and developed numerous long, slender often branching process that tended to form an interconnecting network with neighboring cells. Though analysis of U251 cells from these later time periods would have demonstrated an even more dramatic morphologic change, these measurements were not undertaken because of difficulties inherent in the analysis of these cell outlines.

Increasing concentrations of NGF not only caused cross-sectional area to decrease, it also resulted in an increase in the number of cells exhibiting well developed processes and the number of processes per cell (Table 1.3) This is illustrated by comparing the untreated control cultures with cells in the 4.0 ug/ml NGF treated groups. In untreated Hs294 cultures only 35% of the cells exhibited well developed processes with an average of 1.4 processes per cell. In contrast, in the 4.0 ug/ml NGF treated cultures 87.6% of the cells exhibited well developed processes with an average of 4.5 processes/cell. Similar results were obtained with the TE671 and U251 cell lines.

Table 1.3: The effect of NGF treatment on the number of processes per cell and the percentage of cells exhibiting well developed processes.

Cell Line	Days Post-NGF Treatment	Dose NGF (ug/ml)	Processes per Cell (± S.E.)	Percentage of Cells with Processes
TE671	3	0.0	0.24 ± 0.10	20.0
		0.25	0.76 ± 0.19	48.0
		0.5	1.04 ± 0.18	68.0
		1.0	1.04 ± 0.21	60.0
		2.0	1.28 ± 0.17	80.0
		4.0	1.44 ± 0.20	84.0
Hs294	6	0.0	1.4 ± 0.38	35.0
		0.25	1.9 ± 0.36	53.7
		0.5	2.4 ± 0.48	56.9
		1.0	2.8 ± 0.41	68.6
		2.0	3.2 ± 0.43	74.3
		4.0	4.5 ± 0.78	87.6
U251	14	0.0	0.9 ± 0.24	61.9
		4.0	9.3 ± 0.57	100.0

Discussion

The induction of a more compact, three-dimensional morphology and the production of an increased number of well developed processes is consistent with the evolution of a more differentiated phenotype. These changes are readily visualized and were illustrated both in the preceding article and in the section on morphology in this appendix. Some of the parameters associated with these morphologic changes were amenable to quantification. Image analysis of these same cell lines provided quantitative information on parameters related to the development of a more differentiated state. The development of a more compact, three-dimensional morphology is accompanied by a decrease in maximal cross-sectional area. Figure 1.10 illustrates the ability of NGF to induce a dramatic decrease in cross-sectional area of these neoplastic cell lines. 4.0 ug/ml NGF decreased the cross-sectional area of these neoplastic cells by approximately 67%. Increasing doses of NGF also induce a dramatic increase in the number of cells bearing processes and the number of processes per cell. Treatment with 4.0 ug/ml NGF resulted in a 3.2 - 10.3 fold increase in the number of processes per cell and a 1.6 - 4.2 fold increase in the number of cells exhibiting well developed processes. An increased number of well developed processes is another manifestation of the development of a more differentiated phenotype.

Results from image analysis support conclusions drawn

from morphologic studies and provide quantitative information demonstrating the ability of NGF to induce morphologic changes indicative of the development of a more differentiated phenotype in these neoplastic cell lines of neurogenic origin.

The effect of NGF treatment on NGFR density in the U251 glioblastoma multiforme cell line

The binding of a ligand to its receptor may result in changes in the level of expression (either up- or down-regulation) of the corresponding receptor (Doherty et al. 1988) or associated receptors (Lazarovici et al. 1987). It has been demonstrated in PC12 cells that NGF causes an up-regulation in the number of NGFR present on these cells (Doherty et al. 1988) and a down-regulation of EGFR in these same cells (Lazarovici et al. 1987). Up-regulation of receptors is a mechanism whereby the cells may become more responsive to the ligand. If up-regulation of NGFR increased the responsiveness of neoplastic cells to NGF, there is the potential to design a treatment strategy to exploit this factor and elicit a more dramatic reverse transforming response to NGF. The ELISA test, used previously to measure receptor density, has the capability to provide information as to the effect of NGF treatment on NGFR density in our cell lines. This method was utilized to determine the effect of NGF on expression of NGFR in the U251 glioblastoma multiforme cell line.

Materials and Methods

Cell counts versus protein concentration was determined from untreated and NGF-treated U251 cultures. The ELISA procedure was run in 96-well microtiter plates (See the preceding paper for details on the ELISA procedure). U251 cells were seeded at a density of 12,500 cells/well (0.2 mls at 6.25×10^4 cells/ml). The following day the medium was exchanged with HL-1. On the third day the following groups were established; HL-1 control, 1.0 ug/ml NGF, 2.0 ug/ml NGF, 4.0 ug/ml NGF. Following two days' exposure to NGF, cells from two of the eight seeded wells per group were dissolved in 1.0 N NaOH for 30 min at 37°C. The protein content was determined using the Coomassie blue technique (Pierce) to obtain data on the cell density in these wells. The remaining cells were fixed in 4% paraformaldehyde and receptor density determined using the ELISA technique described in the preceding paper.

Results

Results from this experiment indicate that NGF treatment causes neither up- or down-regulation of NGFR in the U251 cell line. Following exposure to NGF for two days, significant differences between the various treated and untreated groups were not apparent (Table 1.4).

Though measurement of the effect of NGF treatment on tumor growth rate was not a primary objective of this

Table 1.4: The effect of NGF treatment on the expression of NGFR on the U251 glioblastoma multiforme cell line.

Concentration of NGF	Density of NGFR ± S.E. (O.D. units/2.5 x 10^5 cells)
0.0 ug/ml	0.201 ± 0.22
1.0 ug/ml	0.195 ± 0.17
2.0 ug/ml	0.184 ± 0.26
4.0 ug/ml	0.192 ± 0.14

experiment, results from this study support previous data indicating that NGF has the ability to decrease the growth rate of neoplastic cell lines in culture. When the various treatment groups were compared using the untreated control as the standard, it was evident that NGF treatment resulted in an incremental decrease in tumor growth rate. Following two days' exposure to NGF, the 1.0 ug/ml group contained 15.2% fewer cells, the 2.0 ug/ml 18.6% fewer and the 4.0 ug/ml group 20.1% fewer cells than untreated control wells.

Discussion

The U251 cell line develops a number of interesting changes in response to NGF. NGF induces cells from this glioblastoma multiforme cell line to develop a more differentiated phenotype and to stop dividing. These effects appear to be highly persistent. In addition, the U251 cell line grows readily in irradiated nude mice. These characteristics make it a logical choice for further investigations to assess the effects of NGF on neurogenic tumor cell lines.

It has been reported that NGF treatment induces the up-regulation of NGFR in PC12 cells (Doherty et al. 1988). Results from this experiment indicate that NGFR are neither up- or down-regulated in the U251 cell line following two days' exposure to NGF. In fact, a significant difference was not identified between the control and any of the NGF-treated

groups. NGFR up-regulation does not appear to be an aspect that can be exploited in the development of a treatment strategy for the U251 glioblastoma multiforme cell line.

U251 cells respond well to NGF therapy. This is readily demonstrated by the morphologic studies and studies evaluating growth rate. Though this experiment was not designed to illustrate the effect of NGF on growth rate, measurements taken during the course of the study once again demonstrate that NGF is capable of diminishing the growth rate of neoplastic cell lines of neurogenic origin.

The length of NGF-exposure required to induce a maximal morphologic response in the Hs294 Malignant Melanoma cell line

During the course of the *in vitro* evaluation of these five human neurogenic tumor cell lines for response to NGF, it was observed that neoplastic cells exposed to NGF would continue to develop progressive morphologic changes even after the NGF-containing medium had been removed from the culture. This was observed during routine morphologic studies and in experiments evaluating the persistent nature of the NGF-induced changes. The progressive development of morphologic changes following withdrawal of NGF was particularly apparent in the U251 cell line. In studies to evaluate the length of time that NGF-induced morphologic changes would persist in its absence, cultures were exposed to NGF for two days and then the NGF-containing medium was removed. Though U251 cultures

were exposed to NGF for only 48 hours, cells continued to develop morphologic changes for an additional 10 days following removal of the growth factor from the culture medium and these changes were identical to those exhibited by cells continuously exposed to NGF.

The purpose of this experiment was to determine the length of NGF exposure required to induce a maximal response in one of the neoplastic cell lines under study. The Hs294 cell line was chosen because it developed prominent morphologic changes in response to NGF, it developed these changes relatively quickly and the cells did not tend to become detached from the culture surface in response to NGF.

Materials and Methods

General culture procedures were as described in the "Materials and Methods" section of the preceding paper. The Hs294 cell line was utilized for these experiments. 12-well cluster dishes were seeded with 1.0 x 10^4 cells (1.0 ml/well). The following day the medium was exchanged with HL-1 serum free medium. On the third day the medium was exchanged with HL-1 containing 2.0 ug/ml NGF in all wells except the HL-1 control well. The NGF containing medium was removed and replaced with HL-1 after: 1, 2, 4, 8, 12, 16, 24, 33, and 48 hours. Two additional groups were also present. A group in which the NGF-containing medium was left in the cultures throughout the entire four days in which morphologic changes

were evaluated and a group in which the NGF-containing medium was replaced daily. Photographs of each group were taken daily through day 4 post-NGF treatment. One hundred cells from each treatment group were evaluated and placed into one of three morphologic categories: (A) amorphous cells (round to irregular, 2-dimensional, amorphous cells devoid of processes); (B) angular cells (sharply angular cells that were more refractile and three dimensional than cells in group A); and (C) stellate cells (small, stellate, three dimensional cells exhibiting numerous short processes).

Results

The Hs294 malignant melanoma cell line develops an interesting spectrum of changes in response to NGF. Untreated control cultures are predominated by a very primitive population consisting of large, flat, amorphous cells. NGF treatment stimulates many of these cells to develop a more differentiated phenotype. This transformation is characterized by the development of a more compact, three-dimensional morphology and the production of an increased number of well develop processes. In progressing to this state, responsive Hs294 cells pass through an intermediate stage characterized by the development of a more compact, three-dimensional, sharply angular morphology.

In response to NGF, 90-95% of the cells in Hs294 cultures develop a more differentiated morphology. 40-50% of the cells

attain the most differentiated state characterized by the development of a stellate morphology. Table 1.5 indicates that, from the 48-hour time period on, the spectrum of morphologic changes were essentially indistinguishable for those cultures exposed to NGF for 16 hours or more. Cultures exposed to NGF for 16 hours exhibited the same percentage of cells in the least differentiated category (amorphous cells) indicating that approximately the same proportion of cells were stimulated to develop a more differentiated phenotype. Hs294 cultures grown in the presence of NGF for 16 hours also exhibited the same number of cells in the most highly differentiated category, the stellate cells. Thus, the data indicates that at a dose of 2.0 ug/ml NGF a maximal response can be attained following 16 hours exposure to NGF. Indeed, an argument could be made that 8-12 hours exposure to NGF is sufficient to induce a maximal morphologic response as these cultures were generally within 5% of the NGF controls in each category.

Though full expression of the morphologic changes took slightly longer at the lower exposure times, a maximal response was attained after limited exposure. Exposure for longer periods of time did not stimulate a higher percentage of cells to develop a more differentiated phenotype or additional morphologic changes.

Table 1.5: Length of NGF exposure required to induce a maximal morphologic response in the Hs294 malignant melanoma cell line.

NGF Exposure	Day 1:			Day 2:		
	[c]Amorph.	[d]Ang.	[e]Stell.	Amorph.	Ang.	Stell
HL-1	75%	20%	5%	75%	10%	15%
[a]NGF	50%	30%	20%	5%	40%	55%
1 hr.	60%	30%	10%	75%	10%	15%
2 hr.	60%	30%	10%	60%	25%	15%
4 hr.	40%	30%	30%	20%	40%	40%
8 hr.	40%	30%	30%	15%	40%	45%
12 hr.	40%	30%	30%	15%	40%	45%
16 hr.	40%	30%	30%	10%	40%	50%
24 hr.	20%	45%	35%	10%	45%	45%
38 hr.	20%	45%	35%	10%	40%	50%
48 hr.	20%	45%	35%	10%	45%	45%
[b]NGF	20%	45%	35%	10%	55%	35%
	Day 3:				Day 4:	
HL-1	55%	40%	5%	45%	45%	10%
[a]NGF	2%	49%	49%	2%	49%	49%
1 hr.	40%	40%	20%	40%	40%	20%
2 hr.	40%	30%	30%	33%	33%	33%
4 hr.	25%	55%	20%	20%	45%	35%
8 hr.	15%	45%	40%	10%	45%	45%
12 hr.	15%	35%	50%	10%	45%	45%
16 hr.	10%	50%	40%	5%	55%	40%
24 hr.	10%	45%	45%	5%	55%	40%
38 hr.	10%	45%	45%	5%	55%	40%
48 hr.	8%	45%	27%	5%	55%	40%
[b]NGF	5%	50%	45%	5%	55%	40%

[a] 2.0 ug/ml NGF, medium was not changed during the course of the experiment.
[b] NGF-containing medium was replaced daily.
[c] Amorphous cells (least differentiated morphology)
[d] Angular cells (intermediate stage of differentiation)
[e] Stellate cells (most highly differentiated morphology)

Discussion

Results from this experiment confirm previous observations which had indicated that continuous exposure to NGF is not necessary for neoplastic cell lines to develop a maximal morphologic response to the growth factor. Indeed, at 2 ug/ml NGF, Hs294 cells required only 8 hours NGF exposure to develop and maintain morphologic changes nearly equivalent to NGF-control cultures and only 16 hours to develop changes identical to cultures that had been continuously exposed to the growth factor for four days. This type of response is not unique to the Hs294 cell line. Both the T9 (Marushige et al. 1987), D54 and U251 cell lines respond in a similar manner, i.e., limited exposure to NGF (48 hours) results in the development of a maximal morphologic response.

Information such as this is vital to understanding the response of neurogenic tumor cell lines to NGF. It is only after data is obtained on the minimum dose required for maximal response, the minimal dose required to elicit a response, and the length of exposure needed to obtain a maximal response, that experiments can be designed to test specific aspects of the response of these cells *in vitro*.

Conclusions; Chapter 1 Appendix

Information presented in this appendix expands on some of the statements made in the preceding paper and describes studies that provide additional insight into the response of these five human tumor cell lines to NGF *in vitro*. *In vitro* studies demonstrate the ability of NGF to reverse some of the transformed properties of neurogenic tumors cell lines. The succeeding chapter will focus on the effect of NGF on the growth rate of many of these same cell lines *in vivo*.

CHAPTER 2

THE USE OF NERVE GROWTH FACTOR AS A REVERSE TRANSFORMING AGENT FOR THE TREATMENT OF NEUROGENIC TUMORS: *IN VIVO* RESULTS

CHAPTER 2

The Use of Nerve Growth Factor as a Reverse Transforming Agent for the Treatment of Neurogenic Tumors: *In Vivo* Results.

Abstract

The rationale behind the evaluation of natural differentiating agents, such as nerve growth factor (NGF), for reverse transforming potential is based on the theory that such compounds may represent a nontoxic means of controlling tumor growth. Previous *in vitro* experiments have shown that NGF is capable of retarding growth and of inducing persistent differentiation of neurogenic tumor cell lines. *In vivo*, NGF is capable of causing a persistent reduction in the number of ethylnitrosourea-induced neurinomas. In this study, the reverse transforming potential of NGF was evaluated by implanting anaplastic glioma and neurinoma cell lines into Fischer rats and selected human glial tumor cell lines into athymic nude mice. Results indicate that NGF is capable of causing a significant decrease in the growth rate of subcutaneous T9 (anaplastic glioma) and clone 16 (anaplastic neurinoma) implants in syngeneic rats. Significantly, NGF treatment was accompanied by adverse effects that were minimal

and transient. NGF treatment of human glial tumor cell lines implanted into athymic nude mice was less effective. Continued tumor growth (although greatly retarded) following NGF treatment and the limited effectiveness of NGF when using the nude mouse model are aspects that require further investigation. These experiments demonstrated that NGF was able to significantly restrain the growth of two neurogenic tumor cell lines in vivo. Results suggest that NGF may eventually prove useful as part of a therapeutic regime for the treatment of tumors of neurogenic origin.

Introduction

Evidence that nerve growth factor (NGF) is capable of reversing some of the transformed properties of neoplastic cells of neurogenic origin continues to accumulate. Endorsement of this concept is all the more convincing because it is supported by a variety of experimental approaches. The initial indication that NGF might possess reverse transforming capabilities comes from an understanding of the physiologic effects of NGF and the nature of reverse transforming agents. Compounds capable of inducing reverse transformation are generally differentiating agents. As NGF functions as a differentiating agent for neural crest-derived cells during development (Levi-Montalcini and Aloe 1985), evaluation of this compound as a reverse transforming agent for anaplastic tumors of the nervous system is warranted.

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Support for this concept has been generated from a number of in vitro studies. These experiments indicated that NGF is able to reverse many of the transformed properties of susceptible tumor cells. Data demonstrates that: 1) NGF is capable of reversing several of the transformed properties of a variety of neurogenic tumor cell lines, including pheochromocytoma, glioma, medulloblastoma, malignant melanoma and neuroblastoma cell lines (Waris et al. 1973; Reynolds and Perez-Polo, 1975; Greene and Tischler, 1976; Reynolds and Perez-Polo, 1981; Jensen 1987; Marushige et al. 1987; Marushige et al. 1989; Matsushima et al. 1990; Kumar et al. 1990; Vinores and Koestner, 1981; Yaeger et al. 1991); 2) NGF treatment consistently results in the development of a more differentiated phenotype in responsive cell lines (Reynolds and Perez-Polo, 1975; Greene and Tischler, 1976; Reynolds and Perez-Polo, 1981; Vinores and Koestner, 1982; Jensen 1987; Marushige et al. 1987; Marushige et al. 1989; Matsushima et al. 1990; Kumar et al. 1990; Vinores and Koestner, 1981; Yaeger et al. 1991); 3) a common consequence of NGF treatment is a reduction in growth rate or complete cessation of growth (Reynolds and Perez-Polo, 1975; Greene and Tischler, 1976; Vinores and Koestner, 1981; Jensen 1987; Kumar et al. 1990; Matsushima and Bogenmann, 1990; Yaeger et al. 1991); and 4) these effects are persistent in a number of tumor cell lines of neurogenic origin (Reynolds and perez-Polo, 1975; Reynolds and Perez-Polo, 1981; Marushige et al. 1987; Yaeger et al.

1991).

In vivo studies further support the reverse transforming ability of NGF. Treatment of rats with NGF either transplacentally before ethylnitrosourea (ENU) exposure or postnatally after ENU exposure caused a reduction in the number of ENU-induced neurinomas (Vinores and Koestner, 1982; Raju et al. 1989). Data from a one-year study indicates that NGF treatment does not simply delay the onset of tumor formation, but results in a persistent suppression of tumor development (Raju et al. 1989). Another line of evidence demonstrating that NGF is effective *in vivo* comes from studies involving intracerebral implantation of F98 anaplastic glioma cells into syngeneic Fischer rats. When F98 cells were pretreated with NGF, treated with NGF following implantation or implanted along with NGF-producing C6 cells, tumor growth rate was diminished, survival time increased and fewer animals developed tumors (Vinores and Koestner, 1980; Vinores and Koestner, 1981). On histologic examination NGF treated tumors had developed a more differentiated morphology (Vinores and Koestner, 1981).

These results offer convincing evidence that NGF is capable of reversing some of the transformed properties of neoplastic cells both *in vitro* and *in vivo*. We have evaluated a number of tumor cell lines *in vitro* and have found that a diverse collection of neurogenic tumor cell lines exhibit reverse transformation in response to NGF (Yaeger et al.

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1991). *In vitro* results and initial *in vivo* data strongly support the need for further investigation in this area, particularly *in vivo*. The aim of this study was to evaluate the response of several neurogenic tumor cells lines, including cell lines of both human and rodent origin, for response to NGF *in vivo*.

Materials and Methods

Stock cultures

Four cell lines were evaluated for response to NGF *in vivo*: two rodent tumor cell lines (the T9 anaplastic glioma line and clone 16, an anaplastic neurinoma clone) and two human cell lines (the D54 mixed glioma and U251 glioblastoma multiforme cell lines). The two human tumor cell lines were generous gifts from Dr. Bigner at Duke University. Each cell line had previously been shown to exhibit a reverse transforming response to NGF *in vitro* (Marushige et al. 1987; Marushige et al. 1989; Yaeger et al. 1991). Stock cultures of the human cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco) containing 20% fetal bovine serum (Sigma), and 4 mM glutamine in a humidified chamber with 8% CO_2 at 37 °C. The rodent cell lines were maintained under similar conditions except in DMEM containing 10% FBS.

Effects of serum on response to NGF

Cultures were seeded in 12-well cluster plates (Corning)

at a density of 3.0 x 10^3 to 2.5 x 10^4 cells/well (1.0 ml/well). Cells were seeded in DMEM + 20% FBS. After 24 hours, the medium was replaced with either HL-1 serum-free medium (Ventrex) or HL-1 containing 10.0%, 1.0% or 0.1% FBS. The following day the medium was again exchanged with either the serum containing medium or serum containing medium pre-incubated with 2 ug/ml 2.5S NGF (Boehringer Mannheim) for one hour. Cultures were observed daily via phase-contrast microscopy for evidence of NGF-induced morphologic changes.

This experiment was duplicated with alpha-2-macroglobulin replacing the FBS. Concentrations of alpha-2-macroglobulin (bovine, Boehringer Mannheim) equivalent to that found in 10.0%, 1.0% and 0.1% serum were utilized. The corresponding concentrations of alpha-2-macroglobulin were 0.9 mg/ml, 0.09 mg/ml, and 0.009 mg/ml respectively.

In addition, an <u>in vitro</u> bioassay was employed to identify whether there was a difference in the ability of mouse versus rat serum (Sigma) to inhibit NGF-induced morphologic changes. A similar protocol to the one outlined above was used. Various concentrations of each serum (0.75, 0.5, 0.25, 0.1 and 0.075%) were added to HL-1. 2.0 ug/ml NGF was pre-incubated with the serum containing medium for one hour before the final medium change. Corresponding controls, without NGF, were employed at each serum concentration. Cultures were observed daily for NGF-induced changes in morphology.

Experiments involving the rodent tumor cell lines

Cultures of T9 cells one day from confluency were trypsinized, washed, and resuspended in DMEM at 8 x 10^5 cells/ml. A Hamilton syringe with 22-gauge needle was used to implant 25 ul (2 x 10^4 cells) of this cell suspension into the subcutaneous tissue of the left flank of 90-110g Fischer 344 rats (Charles River). Tumors were allowed 10 days to become established before treatment was initiated. The various NGF (2.5S Boehringer Mannheim) treatment regimes employed are described in the Results section. Animals were weighed once a week. Tumors were palpated and measured twice weekly with vernier calipers. Animals were euthanized with CO_2 once the tumors had reached a maximum of 1.0-1.5 cm in any one dimension. Samples of each tumor were fresh frozen, fixed in 4% paraformaldehyde for 16 hours and then frozen, and fixed in 10% neutral buffered formalin. Routine histologic sections were examined from each tumor.

Procedures involving clone 16 implants were performed in a similar manner. The implant dose was decreased slightly (3.5 x 10^5 cells/implant) for these experiments and the tumors were generally allowed 7 days to become established before treatment was initiated.

Experiments involving the human tumor cell lines

The implantation procedure was modified slightly for the human tumor lines. Four-week-old athymic nude mice (Harlan

Sprague Dawley) were irradiated (gamma irradiator) with 322R on the day of implantation. Cultures of U251 cells one day from confluency were trypsinized, washed and resuspended in DMEM. 0.25 mls (7.9×10^6 cells) of this cell suspension was implanted into the subcutaneous tissue of the dorsal midline between the shoulder blades. Tumors were allowed 7 days to become established before treatment was begun.

Tumors from D54 implants developed slowly and inconsistently. As such, the D54 cell line was passaged three times in irradiated athymic nude mice to obtain an animal-adapted population of cells. These cells, when re-established in culture, continued to exhibit a reverse transforming response to NGF. Following the third passage, tumors were aseptically removed from 4 animals, minced, passed through 40- and 100-mesh cytosieves, washed and then a standard volume (0.25 mls) was implanted into each experimental animal. Tumors were allowed 7 days to become established before treatment was begun.

NGF-receptor studies

NGF receptor (NGFR) density was determined for each cell line using a modification of the ELISA procedure described by Doherty et. al. (1988). Cells were seeded at 15,000 cells/well in a 96-well microtiter plate. Following two days in culture, the cells were fixed with paraformaldehyde. Receptor density was determined using ME20.4, a human anti-

NGF-receptor antibody (1:20, Amersham) or Mab 192, a rat anti-NGF-receptor antibody (1:200, Oncogene Science) and an anti-mouse horseradish peroxidase conjugate (1:1000, Sigma). Optical density (OD) was measured with the Dynatech MR600 microtiter plate reader. A standard curve was developed using two cell lines with known NGF-binding properties, the human Hs294 malignant melanoma cell line (Fabricant et al. 1977) and the rodent PC12 pheochromocytoma cell line (Sonnenfeld and Ishii, 1985; Azar et al. 1990). OD was determined at various seeding densities for each standard. Protein concentrations were determined from which cell numbers were calculated. A standard curve was generated that compared OD to Hs294 or PC12 cell numbers. Unknowns were then compared to the standard curve.

Results

As a preliminary screen, the NGFR density of each tumor cell line was estimated using an ELISA. Results indicated that all four tumor lines used in the in vivo experiments (T9, clone 16, D54 and U251) had very few NGFR compared to the two control cell lines (Hs294 and PC12). Cell lines were compared on an arbitrary scale (1.0 arbitrary unit corresponds to 0.165 O.D. units per 2.5 x 10^4 cells.). The positive control for the human tumor lines, the Hs294 malignant melanoma line, exhibited a receptor density (106.7 $\pm$ 12.36) that was nearly two orders of magnitude higher than either the D54 (1.7 $\pm$

0.34) or U251 (1.4 ± 0.17) cell lines. The PC12 cell line (85.9 ± 10.01), the positive control for the rodent tumor cell lines, also exhibited a receptor density that was approximately two orders of magnitude higher than either clone 16 (0.27 ± 0.13) or the T9 (0.91 ± 0.33) cell line. Despite low receptor numbers, each cell line responded well to NGF in vitro (Marushige et al. 1987; Marushige et al. 1989; Yaeger et al. 1991), albeit at high concentrations of NGF.

Initial experiments, in which NGF was administered subcutaneously at a site distant from the T9 anaplastic glioma implants, demonstrated an encouraging trend. Nerve growth factor treatment resulted in a statistically significant ($P < 0.05$) decrease in the average volume of subcutaneous tumor implants in the high dose NGF-treatment group (40 ug NGF SID for 5 days) compared to saline-treated controls. At the time of necropsy the average tumor volume in the high dose NGF-treated group (12.62 ± 2.02 mm^3/day) was less than half that of the saline-treated controls (27.72 ± 4.52 mm^3/day). The average tumor volume in the low dose NGF-treated group (10 ug SID for 5 days) was decreased slightly (24.14 ± 6.30 mm^3/day) compared to the controls, but this difference was not statistically significant.

Following these favorable preliminary results, an attempt was made to develop a more effective treatment protocol. Various doses and dosage schedules were evaluated. Of these regimes, 35 ug NGF administered directly at the implantation

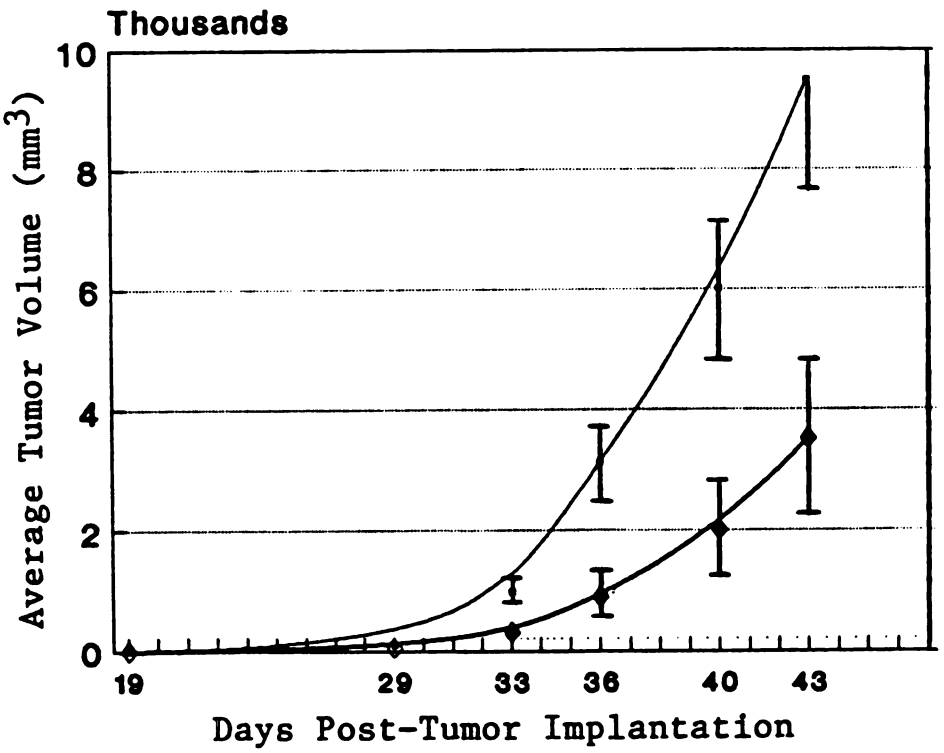
site once a day on 6 consecutive days (210 ug total dose) yielded the most favorable results.

A full scale experiment was performed using this treatment protocol. Results are shown in figure 2.1. Treatment with this schedule resulted in a statistically significant ($P < 0.05$) decrease in the growth rate of subcutaneous T9 implants in the NGF-treated group compared to the untreated controls. A significant difference was identified at each measurement period once tumors could be reliably palpated. At the time of necropsy, the average tumor volume in the NGF-treated group was only 37% that of the untreated controls.

Similar experiments were conducted with clone 16. Data from clone 16 experiments demonstrated that NGF (40ug once-a-day (SID) for 6 days) was even more effective at diminishing the growth rate of subcutaneous neurinoma implants (figure 2.2). As with the T9 implants, a significant difference was identified at each measurement period once tumors could be reliably palpated. At the time of necropsy, the average tumor volume in the NGF-treated group was only 27% that of the untreated controls.

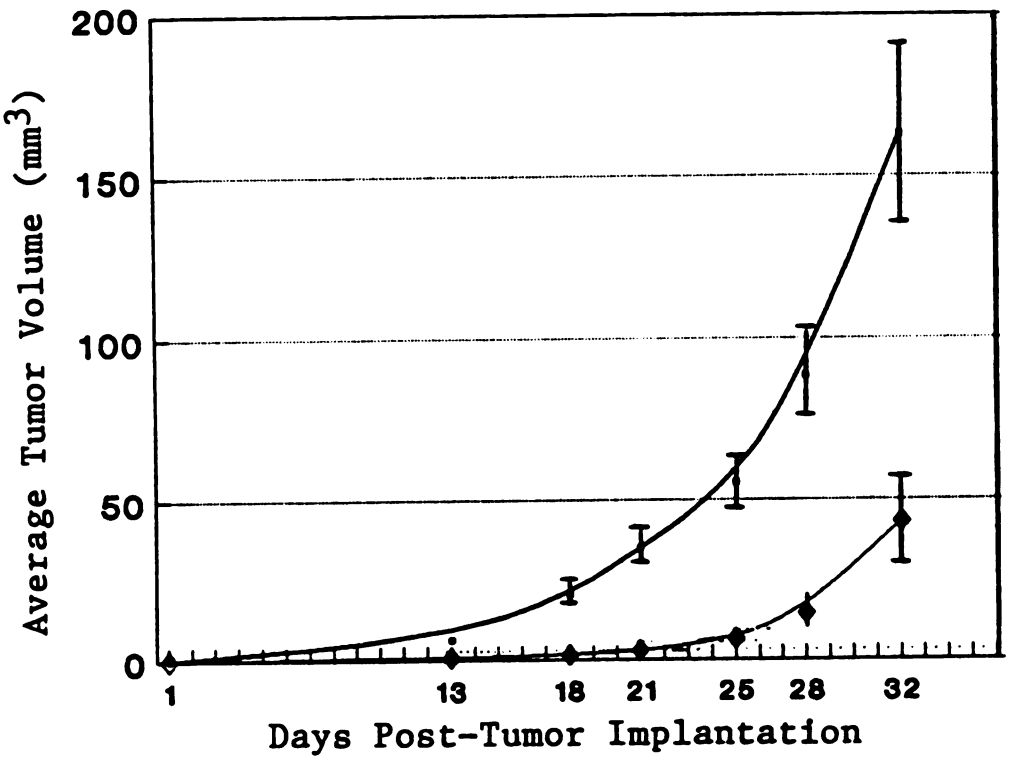
With both T9 and clone 16 implants, NGF treatment resulted in a significant decrease in tumor growth rate. However, tumors in the NGF-treated groups continued to grow despite therapy. These findings prompted an additional experiment. A second series of NGF injections (40 ug NGF SID,

Figure 2.1: The effect of NGF treatment on the growth rate of subcutaneous T9 anaplastic glioma implants.



35 ug NGF was administered directly at the implantation site SID on days 10-15 post-implantation (210 ug total dose). Results indicate that the average tumor volume in the NGF-treated group was decreased significantly compared to saline-treated controls from day 33 on. Each group contained ten experimental subjects.
(•) NGF-treated group
(•) saline-treated controls

Figure 2.2: The effect of NGF treatment on the growth rate of subcutaneous clone 16 anaplastic neurinoma implants.



40 ug NGF was administered directly at the implantation site SID on days 10-15 post implantation (240 ug total dose). Results indicate that the average tumor volume in the NGF-treated group was decreased significantly compared to saline-treated controls from day 18 on. Each group contained seven experimental subjects.
(•) NGF-treated group
(•) saline-treated controls

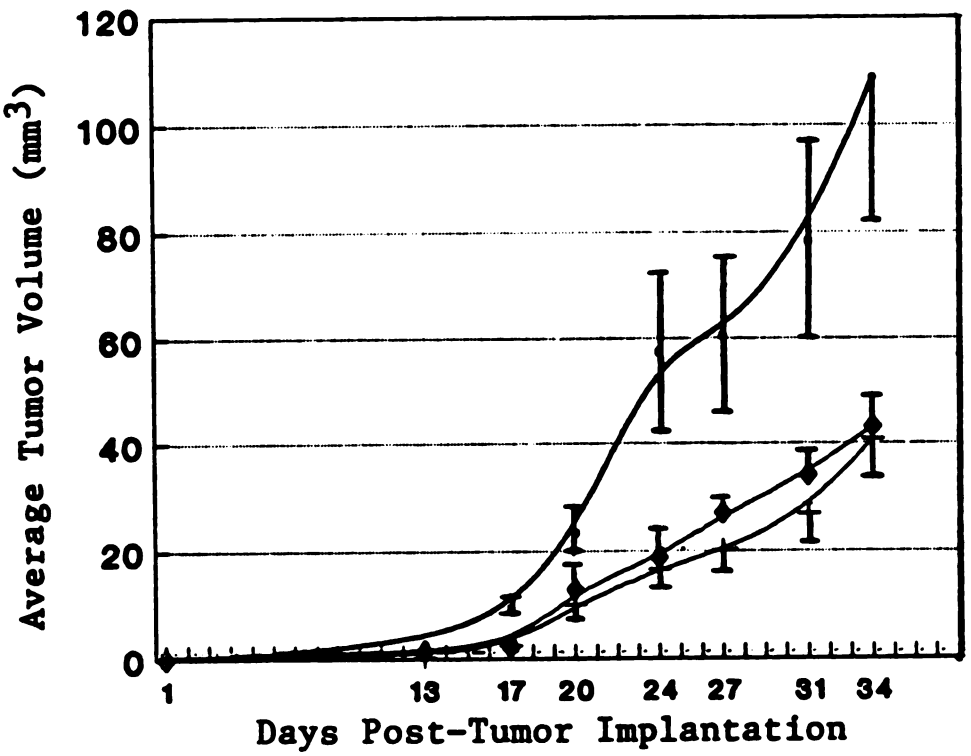
days 8-12 & 22-26) was administered to determine if repeated series of treatments would result in an additional decrease in tumor growth rate. This second series of NGF-treatments had no additional effect on tumor growth (Figure 2.3), even though the tumors were still responsive at the time of the second injection period (data not shown).

Experiments to determine the effect of NGF treatment on two human tumor lines, in vivo, were also undertaken. In contrast to the rodent tumor cell lines, only a minimal response to NGF (35 ug NGF SID for 6 days) was observed following treatment of these tumors in nude mice. In each case, NGF treatment resulted in a slight decrease in growth rate (data not shown). However, the decrease was not nearly as dramatic as with the rodent tumor lines and was not statistically significant.

Reports indicate that maturation factors are capable of inducing a phenotypic change in vitro (Waris et al. 1973; Reynolds and Perez-Polo, 1975; Greene and Tischler, 1976; Reynolds and Perez-Polo, 1981; Jensen 1987; Marushige et al. 1987; Marushige et al. 1989; Matsushima et al. 1990; Kumar et al. 1990; Vinores and Koestner, 1981; Yaeger et al. 1991) as well as in vivo (Vinores and Koestner, 1981). All tumors in this study were examined histologically. Differences in morphology were not identified between NGF-treated and untreated tumors.

NGF-treated animals exhibited a transient decreased rate

Figure 2.3: The effect of a repeated series of NGF treatments on the growth rate of clone 16 implants.



Results demonstrate that the average tumor volume in both NGF-treated groups was decreased significantly compared to saline-treated controls. However, the growth rate of the group receiving a repeated series of NGF treatments was not significantly different from the group that received a single series of NGF-injections. Each group contained seven experimental subjects.
(•) saline-treated control group;
(+) single series of NGF injections (40 ug NGF SID days 8-12);
(•) repeated series on NGF treatments (40 ug NGF SID days 8-12 & 22-26).

of weight gain compared to saline treated controls. However, the animals recovered rapidly, and, within one week following cessation of NGF injections, there were no differences in the weights between the various groups. This was the only evidence of adverse consequences of NGF therapy. Histologic evidence of organ damage was not identified. Outgrowths of neurons were not apparent at the treatment site and intracerebral inoculation was without noticeable effect on cerebral architecture.

The results from several in vitro studies are reported here because they provide an insight into potential causes of the discrepancy between implantation studies involving rodent and human tumor cell lines. These data illustrate the effects of serum on the response of tumor cell lines to NGF in tissue culture. Tumor cell lines fail to exhibit a morphologic response to NGF in the presence of 10% and 1.0% FBS but will respond at concentrations $\leq$ 0.1% (Table 2.1). However, even in the presence of only 0.1% serum, the NGF-induced morphologic changes were diminished compared to corresponding controls. When levels of alpha-2-macroglobulin, the major NGF-binding protein in serum (Ronne et al. 1979), equivalent to those present at the various serum concentrations were added to the cultures, similar results were obtained (Table 2.1). In addition, both rat and mouse sera were evaluated for their ability to prevent NGF-induced morphologic changes. These experiments indicate that mouse serum and FBS are

Table 2.1: Comparison of FBS, alpha-2-macroglobulin, mouse and rat serum in terms of their ability to inhibit or diminish NGF-induced morphologic changes in the D54 mixed glioma cell line.

Days Post-NGF administration	1	2	3	4	5	6	7	8	9	10
% FBS										
10.0%	0	0	0	0	0	0	0	0	0	0
1.0%	0	0	0	0	0	0	0	0	0	0
0.1%	0	0	0	0	+	+	+	+	+	+
alpha-2-macroglobulin (mg/ml)										
0.9	0	0	0	0	0	0	0	0	0	0
0.09	0	0	0	0	0	0	0	0	0	0
0.009	0	0	0	0	+	+	+	+	+	+
% mouse serum										
0.75%	0	0	0	0	0	0	0	0	0	0
0.5%	0	0	0	0	0	0	0	0	0	0
0.25%	0	0	0	0	0	0	0	0	0	0
0.1%	0	0	0	0	+	+	+	+	+	+
0.075%	0	0	0	+	+	2+	2+	2+	2+	2+
% rat serum										
0.75%	0	0	0	0	0	0	0	0	0	0
0.5%	0	0	0	0	+	+	+	+	+	+
0.25%	0	0	0	+	+	2+	2+	2+	2+	2+
0.1%	0	0	+	+	2+	2+	2+	3+	3+	3+
0.075%	0	+	+	2+	2+	2+	3+	3+	4+	4+

2.0 ug/ml NGF was pre-incubated with each concentration of serum or alpha-2-macroglobulin for one hour prior to exchange with the culture medium. The degree of change was graded from 0 (no difference from untreated controls) to 4+ (majority of cells exhibiting NGF-induced changes in morphology).

approximately equivalent in their ability to diminish NGF-induced changes in morphology. In contrast, rat serum appears to be only one-fifth as potent in its ability to prevent or diminish NGF-induced morphologic changes (Table 2.1).

Discussion

In vivo experiments utilizing the two cell lines of rodent origin clearly demonstrate that NGF treatment is capable of causing a dramatic decrease in the growth rate of subcutaneous T9 (anaplastic glioma) and clone 16 (anaplastic neurinoma) implants. At the various measurement periods, the average tumor volume in the NGF-treated group was only 8-27% that of saline-treated controls for clone 16 implants and only 29-37% that of saline-treated controls for T9 implants. This confirms the results from *in vitro* studies which demonstrated that NGF was able to reverse several of the transformed properties of these tumor cell lines (Marushige et al. 1987; Marushige et al. 1989). These results are all the more encouraging because adverse effects associated with NGF administration in experimental animals were minimal and transient.

Based on the promising results obtained with the rodent tumor lines *in vivo* and the dramatic response of the human tumor cell lines to NGF *in vitro* (Yaeger et al. 1991), both prior to implantation and once re-established from the implants, it was expected that a similar decrease in tumor

growth rate would be observed with the human tumor lines following NGF treatment *in vivo*. Implants of the human cell lines displayed a slight decrease in growth rate in response to NGF. However, the magnitude of response was not nearly as great as with the rodent tumor lines and this decrease was not statistically significant. A complete explanation for the difference between the two model systems was not established although several factors were identified that may have contributed to this disparity. The presence of serum or alpha-2-macroglobulin in the culture medium is capable of diminishing or preventing NGF-induced morphologic changes *in vitro* (Table 2.1). Additionally, data indicate that mouse serum is more potent than rat serum in its ability to diminish the NGF-induced response (Table 2.1). These factors become significant when combined with the observation that slight hemorrhage and/or serum transudation was not uncommon following injections in the athymic nude mice due to the delicate nature of their subcutaneous tissues. Rats, in which these experiments were highly successful, did not exhibit such a tendency. It is plausible that serum present at the time of NGF-administration inhibited the NGF-induced response in the athymic nude mice and may have played a key role in the disparity between the two systems.

The inherent difficulties encountered with the athymic nude mice were not a factor in studies involving the implantation of rodent tumor cell lines into syngeneic Fischer

rats. These experiments clearly demonstrate the ability of NGF to reduce the growth rate of subcutaneous tumor implants. However, the matter of continued tumor growth in NGF-treated groups requires further analysis before definitive conclusions can be made as to the potential of this type of therapy. It does not appear that continued tumor growth is simply the result of a transient response to NGF. If the effects of NGF were transient, the second series of NGF-treatments should have elicited an additional response. The fact that this was not observed argues against such an interpretation. A plausible hypothesis, and one that is supported by several lines of experimental evidence, is that NGF causes persistent growth suppression in a NGF-responsive subpopulation of cells and that an NGF-insensitive subpopulation of cells accounts for continued growth of the tumors. The therapeutic potential of NGF may hinge on the basis of these findings. If a transient suppression of growth is all that can be attained, then the therapeutic potential of NGF would be rather limited. However, if NGF is capable of causing persistent growth suppression in responsive cells, it may prove useful as a reverse transforming agent.

The rationale behind evaluating natural differentiation agents for reverse transforming potential is based on the theory that these compounds may represent a nontoxic means of controlling tumor growth. Our data indicate that NGF is capable of causing a dramatic decrease in the growth rate of

subcutaneous T9 (anaplastic glioma) and clone 16 (anaplastic neurinoma) implants. Significantly, this decreased tumor growth rate is accompanied by adverse effects that are minimal and transient. Further study is needed to answer questions concerning the mechanism responsible for this growth suppression and whether or not these effects are persistent in vivo. None-the-less, it is clear that NGF is able to suppress the growth of rapidly dividing neoplastic cell lines of neurogenic origin. We are optimistic that this compound may prove useful, alone or in combination with other types of therapy, for the treatment of tumors of neurogenic origin.

CHAPTER 2 APPENDIX

Introduction

The preceding paper provides information supporting the ability of NGF to reverse some of the transformed properties of implanted neurogenic tumor cell lines. As with the *in vitro* studies, the material presented in the article did not include all of the information obtained from *in vivo* studies. The scope of the paper was limited to information necessary to demonstrate the reverse transforming capabilities of NGF *in vivo* and to discuss some of the pitfalls associated with these types of experiments. Additional *in vivo* studies were performed. The results of these experiments are reported here, in the appendix, because they provide additional information on techniques involved in tumor implantation studies and on some of the difficulties encountered in the course of this line of experimentation.

Two approaches were utilized for the *in vivo* studies. The first approach was to implant tumors subcutaneously. The advantage of this method is that the tumors are readily palpable and can be measured at regular intervals. This provides information on tumor growth rate. The disadvantage of this method is that the treatment is not evaluated in the

environment in which it will eventually be used. The second approach was to implant tumors intracerebrally. The advantage of this method is that treatment is evaluated in the environment in which the tumors naturally occur. The drawback to the intracerebral studies is that measurements of tumor size between the time of implantation and necropsy are difficult unless animals are sacrificed periodically throughout the course of the experiment or expensive methods, such as Magnetic Resonance Imagery (MRI), are employed. Initial experiments concentrated on subcutaneous studies because they allowed tumor growth rate to be evaluated and these studies were complicated by fewer technical difficulties. These experiments were designed to; 1) determine whether the tumor cell lines would respond to NGF in vivo, 2) develop an optimum treatment protocol and 3) to obtain measurements on the effect of NGF on tumor growth rate. The results of the subcutaneous experiments are detailed in the preceding paper. The subcutaneous experiments were followed by intracerebral studies to evaluate NGF treatment in the environment in which the tumors would normally be found. Results from the intracerebral studies and additional information on some of the subcutaneous experiments are outlined in this appendix.

Experiment A: A preliminary study of the behavior of intracerebral and subcutaneous T9 anaplastic glioma implants

Experiment A was a preliminary study designed to develop effective subcutaneous and intracerebral implantation procedures and to obtain baseline information on the growth of T9 anaplastic glioma implants in syngeneic Fischer 344 rats.

Materials and Methods

Ten, 90-110g Fischer 344 rats were anesthetized with 0.9 mls of a 3:1 mixture of PromAce (Fort Dodge 10mg/ml) and Ketaset (Bristol 100mg/ml) administered intraperitoneal (IP). The dorsum of the skull was shaved and swabbed with alcohol. A hole was drilled through the skull with a 20 gauge needle at the intersection of a line drawn directly caudal from the medial canthus of the left eye and a line drawn between the openings of the external ear canals. A 50 ul Hamilton syringe equipped with a 22 gauge needle was used to implant the T9 suspensions. The needle was inserted to a depth of 4mm below the surface of the skull. These measurements were developed with the aid of cadaver specimens and were designed to implant the cell suspensions into the left parietal lobe. 10^5 T9 cells in 25 ul DMEM-0 was injected into this location. An equal volume of this suspension was implanted into the subcutaneous tissue of the right lateral thorax. Animals were observed twice a day and were euthanized once they began to

exhibit progressive neurologic signs. At necropsy, the subcutaneous tumors were measured with vernier calipers. Sections of both the subcutaneous and intracerebral tumors were fresh frozen, fixed in 4% paraformaldehyde for 16 hours and then frozen, and fixed in 10% neutral buffered formalin. Routine histologic sections were evaluated from the subcutaneous and intracerebral tumors. The brain was sectioned at the level of the implantation site, and 4.0 mm cranial and caudal to the implantation site. The brain stem and cerebellum were sectioned sagittally 2 mm lateral to the median plane.

Results

Rats receiving intracerebral implants generally exhibited the following progression of clinical signs. The animals would first develop a reddish oculonasal discharge (chromodactoria). This was followed by depression and weight loss. Eventually, rats would develop a variety of neurologic signs including progressive ataxia, circling, and posterior paresis. Animals were euthanized once progressive neurologic signs became apparent. 90% (9 of 10) of the rats developed intracerebral tumors and 80% (8 of 10) developed subcutaneous tumors following implantation of T9 suspensions. The average survival time of animals that developed intracerebral tumors was 31.4 $\pm$ 1.4 days. The average volume of the subcutaneous implants at the time of necropsy was 1439 mm^3.

On histopathologic examination, the tumors consisted of an invasive population of large spindle-shaped cells arranged in intertwining streams and bundles with minimal supporting stroma. These spindle cells had moderate to abundant pale eosinophilic cytoplasm and indistinct cytoplasmic boundaries. Marked variation in nuclear size and morphology was evident. The large oval nuclei generally had coarsely clumped chromatin with 1-3 variably sized, often large, irregular nucleoli. Two to four, at times bizarre, mitotic figures were observed per HPF. The invasive tendencies, marked pleomorphism, relatively high mitotic index and rapid growth rate suggest that tumors derived from T9 implants represent a highly anaplastic neoplasm.

Though 9 of 10 rats developed intracerebral tumors following implantation of T9 cell suspensions, only 4 of these animals had tumors present at the implantation site in the left parietal lobe. In all 9 cases, tumor was apparent in the cerebellum. Variably sized ribbons and nodules of neoplastic cells were present between the cerebellar folia. The neoplastic proliferation was invasive and frequently extended into the cerebellar white matter. Isolated vessels within the molecular layer were surrounded by cuffs of tumor cells. Nodules of neoplastic cells were frequently identified at the junction of the cerebellum and brainstem and involving the area between the dorsal cerebellum and cerebral hemispheres. In 8 of 9 rats the neoplastic proliferation was also apparent

along the ventral portions of the brainstem.

Discussion

This preliminary experiment demonstrated that both subcutaneous and intracerebral tumors could be consistently established in Fischer 344 rats following the implantation of suspensions of T9 anaplastic glioma cells. This study also provided information on the time frame for tumor development and the progression of clinical signs. It also illustrated several pitfalls. The two major problems identified were inconsistent tumor development at the intracerebral implantation site and widespread submeningeal dissemination of neoplastic cells throughout the cranial vault. Neoplastic cells would leak from the implantation site and settle in locations where spacial factors favored growth or along ventral portions of the brain. This resulted in tumors forming around the cerebellum and along the ventral portions of the brainstem.

We were encouraged by the high rate at which tumors developed following implantation both intracerebrally and subcutaneously, but were disappointed with the lack of confinement of the intracerebral implants to the implantation site. In order for meaningful information to be gained from future experiments, methods to achieve more consistent development of tumors at the intracerebral implantation site and to minimize tumor dissemination throughout the cranial vault would have to be developed.

Experiment B: The effect of NGF treatment on the growth of subcutaneous and intracerebral T9 anaplastic glioma implants

The focus of this experiment was to try and refine the implantation procedure used in the previous experiment and to evaluate the response of intracerebral and subcutaneous T9 implants to NGF.

Several modifications were made to our original implantation procedure to try and alleviate some of the problems identified in Experiment A. A guide was developed to better control the depth at which the implants were seeded. The implants were seeded slightly deeper into the cerebral parenchyma to try and confine the tumors to the implantation site. Also, the needle was left in position for 30 seconds following implantation in an attempt to diminish the leakage of neoplastic cells from the implantation site.

Materials and Methods

A protocol similar to the one described for Experiment A was used for this study, with the following modifications. A depth gauge was developed to increase implant placement accuracy. An 18 gauge needle was cut at a level which allowed the 22 gauge needle, when passed through the guide, to extend 5 mm below the end of the guide when it was positioned on the dorsum of the skull. The tumors were implanted to a depth of 5 mm, compared to 4 mm in the previous study. The implantation depth was increased in an attempt to confine the

tumors to the implantation site. The needle was then left in place for 30 seconds to try and diminish pressure induced escape of cells along the needle tract.

The implants were allowed 10 days to become established before NGF treatment was begun. Rats were divided into three groups containing 10 animals each. All treatments were administered subcutaneously (left flank) at a site distant from either implant. The control group received 0.4 ml of the final dialysate solution (10mM sodium acetate + 15 mM sodium chloride). The low dose treatment group received 10ug NGF (0.1 ml) dissolved in 0.9% saline and administered once a day (SID) for five days. The high dose treatment group received 40 ug NGF (0.4 mls) SID on five consecutive days. Animals were observed twice a day and were euthanized once they began to exhibit progressive neurologic signs. At necropsy, both the subcutaneous and intracerebral tumors were measured with vernier calipers. Sections of the subcutaneous and intracerebral tumors were fresh frozen, fixed in 4% paraformaldehyde for 16 hours and then frozen and fixed in 10% neutral buffered formalin. Routine histologic sections from the subcutaneous and intracerebral tumors were evaluated as per Experiment A.

Results

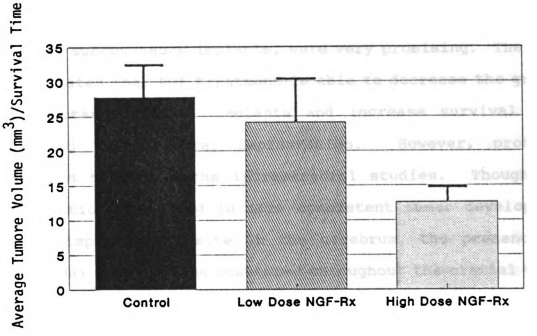
The average survival time for animals in the high dose NGF treatment group (200 ug NGF total dose) was 32.4 $\pm$ 0.8

days, for the low dose NGF treatment group (50 ug NGF total dose) was 28.6 ± 0.8 days and for the untreated control was 29.3 ± 1.0 days. The value of the data on survival time is questionable due to the variability in tumor development as a result of widespread submeningeal dissemination of the neoplastic cells.

Compared to the effect on survival time, the effect of NGF treatment on the growth of subcutaneous tumor implants was striking. The average tumor volume (average tumor volume at the time of necropsy/survival time) in the high dose NGF-treated group was less than half that of the average tumor volume of the untreated control (Figure 2.4). Interestingly, only seven (7 of 10) animals in the high dose treatment group developed subcutaneous tumors compared to 9 (9 of 10) for the low dose NGF treatment group and all 10 in the untreated control group.

The changes made in the implantation procedure greatly increased the number of tumors present at the implantation site. 87.9% (29/33) of the rats developed tumors at the implantation site compared to only 44.4% (4/9) in Experiment A. However, these tumors were not well confined to the implantation site. Neoplastic cells had leaked from the implantation site and seeded other areas of the brain including the cerebellum and brainstem. 81.8% (27/33) of the animals exhibited cerebellar tumors and 75.8% (25/33) of the animals exhibited brainstem involvement. In addition, T9

Figure 2.4: The effect of NGF treatment on the growth of subcutaneous T9 implants.



Results are reported as average subcutaneous tumor volume at the time of necropsy divided by survival time (mm^3/day). Results are described in these units because of variations in survival time between individual animals. The high dose NGF treatment group (200 ug NGF) exhibited a significant decrease ($P < 0.05$) in the average tumor volume at the time of necropsy (12.6 ± 2.02 mm^3/day) compared to the untreated control group (27.7 ± 4.52 mm^3/day). The low dose NGF treatment group (50 ug NGF) exhibited a slight decrease in tumor growth (24.1 ± 6.30 mm^3/day), however, this difference was not statistically significant.

implants exhibited a tendency to form cap-like masses covering the parietal lobe above the implantation site (42.4%, 14/33).

Discussion

The results from these experiments, particularly those from the subcutaneous implants, were very promising. The data demonstrates that NGF treatment is able to decrease the growth of subcutaneous tumor implants and increase survival time following intracerebral implantation. However, problems continued to plague the intracerebral studies. Though the modifications resulted in more consistent tumor development at the implantation site in the cerebrum, the presence of additional tumor masses scattered throughout the cranial vault continued to plague the procedure. The variability in tumor development undermined the value of the intracerebral studies. Differences in survival time of individual animals was not exclusively a reflection of treatment, it was also dependent on the variability in tumor development.

This experiment was the initial study in which the effect of NGF treatment was evaluated *in vivo*. The dramatic decrease in the growth of subcutaneous implants following NGF treatment indicates that this form of therapy may offer significant therapeutic potential. However, several aspects of the experimental procedure need to be improved. If intracerebral experiments are to be continued, a better method of localizing the tumors to the implantation site needs to be developed.

This problem is addressed in future studies (Experiment E).

Experiment D: An evaluation of the effects of various NGF treatment regimes on the growth rate of subcutaneous T9 implants

The previous experiment, Experiment B, demonstrated that NGF treatment was able to significantly reduce the growth rate of subcutaneous T9 anaplastic glioma implants. It was also apparent from the results that there was a significant difference between the efficacy of the two treatment regimes. 200 ug NGF total dose was far more effective at suppressing tumor growth than the 50 ug total dose. Therefore, before another large scale experiment evaluating the therapeutic potential of NGF was initiated, it was deemed prudent to try and optimize the treatment protocol. Experiment D was designed to evaluate the effectiveness of various NGF treatment doses and dosage schedules when injected directly into the site of the subcutaneous T9 tumor implant.

Materials and Methods

A suspension containing 10^4 T9 cells in 25ul (4 x 10^5 cells/ml) was injected into the subcutaneous tissue of the right caudolateral thorax of 90-110g Fischer 344 rats. Rats were palpated eight days following implantation at which time 88% had palpable nodules at the implantation site. Treatment was begun ten days following implantation. Rats were

restrained in lateral recumbency, the tumor palpated and the NGF injected directly into the region of the tumor. 42 rats were divided into 7 groups containing 6 animals each. The following treatments were administered. Time was measured from the date of implantation.

Group #1) (control 1): 0.1 ml of the dialysate, day 10.
Group #2) 35ug NGF (35ug/0.1 ml), day 10, 35ug total dose.
Group #3) 35ug NGF (35ug/0.1 ml), days 10-13, 105ug total dose.
Group #4) 35ug NGF (35ug/0.1 ml) administered twice a day at a 1/2 hour interval, days 10-13, 210ug total dose.
Group #5) 35ug NGF (35ug/0.1 ml), days 10-16, 210ug total dose.
Group #6) 70ug NGF (70ug/0.2 ml), days 10-16, 420ug total dose.
Group #7) (control 2): 0.1 ml of the dialysate, days 10-16.

The subcutaneous tumors were measured weekly with vernier calipers and tumor volume was calculated. Animals were euthanized with CO_2 once the tumors had reached a maximum of 1.0-1.5 cm in any one dimension. Samples of each tumor were fresh frozen, fixed in 4% paraformaldehyde for 16 hours and then frozen, and fixed in 10% neutral buffered formalin. Routine histologic sections were examined from each tumor.

Results

Though the results from this experiment were not clear-cut, useful information was gained from this study. It is apparent from table 2.2 that fewer animals in the NGF treated groups developed tumors. Because there were so few animals in each group, comparing individual groups was not very rewarding. However, when the percentage of rats developing

Table 2.2: The percentage of rats free from palpable subcutaneous nodules following NGF treatment

Days Post-Implantation	day 29	day 36	day 43	day 48 (necropsy)
Untreated	13.33%	33.0%	33.0%	26.7%
NGF treated	26.7%	50.0%	43.4%	40.0%

This table illustrates the percent of rats free from palpable tumor nodules following subcutaneous T9 anaplastic glioma implants. These values are the product of combining the two control groups and are compared to the combined NGF-treated groups.

tumors in the combined NGF treated groups was compared with that of the combined control groups, it was apparent that fewer tumors developed in animals treated with NGF.

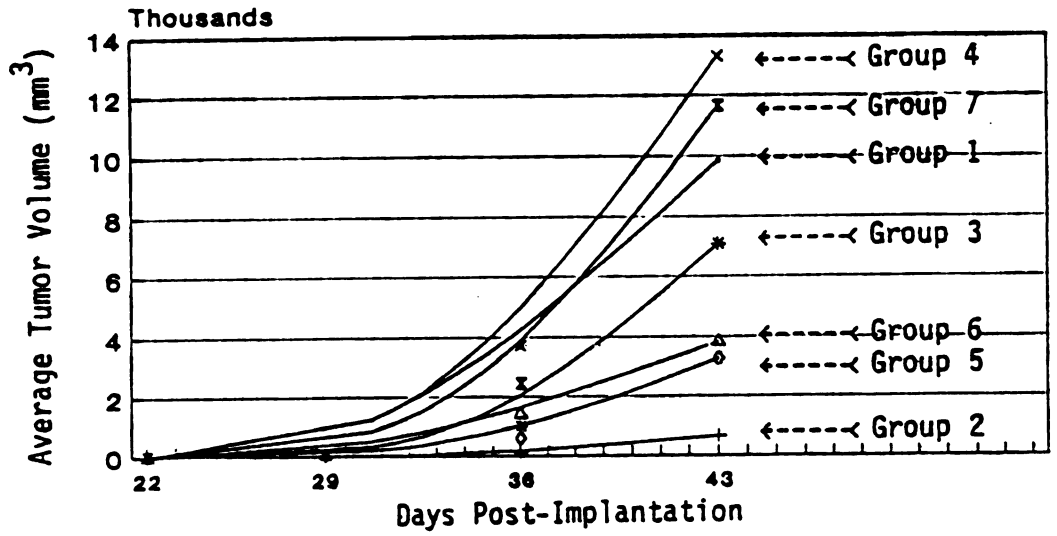
It is evident from figure 2.5 that NGF treatment suppressed the growth rate of subcutaneous T9 implants. Four of the five NGF-treated groups exhibited a decrease in growth rate of subcutaneous T9 implants compared to the untreated controls. This experiment once again demonstrates the ability of NGF to diminish the growth rate of neurogenic tumors *in vivo*. However, a treatment protocol that was definitively superior was not established. Despite the lack of an unambiguously optimum protocol, the results demonstrate that 210 ug NGF divided over a 6 day period is an effective therapeutic regime.

On histologic examination morphologic differences between NGF-treated and untreated tumors were not apparent.

Discussion

Results from this experiment were both encouraging and puzzling. It is difficult to explain the results from Group 4. Animals in this group received a total dose of 210 ug NGF. This was the same total dose administered to rats in Group 5 given on a schedule similar to that of Group 3. Both these groups exhibited a decrease in tumor growth rate in response to treatment, yet the animals in Group 4 had an average tumor volume that was greater than either control group. In light

Figure 2.5: The effect of various NGF doses and dosage schedules on the growth rate of subcutaneous T9 implants.



This graph illustrates the effects of various NGF treatment regimes on the growth rate of subcutaneous T9 implants. The following treatments were evaluated:

Group #1) (control 1): 0.1 ml of the dialysate, day 10.
Group #2) 35ug NGF (35ug/0.1 ml), day 10, 35ug total dose.
Group #3) 35ug NGF (35ug/0.1 ml), days 10-12, 105ug total dose.
Group #4) 35ug NGF (35ug/0.1 ml) administered twice a day at a 1/2 hour interval, days 10-12, 210ug total dose.
Group #5) 35ug NGF (35ug/0.1 ml), days 10-15, 210ug total dose.
Group #6) 70ug NGF (70ug/0.2 ml), days 10-15, 420ug total dose.
Group #7) (control 2): 0.1 ml of the dialysate, days 10-15.

Four of the five NGF-treated groups exhibited a decrease in growth rate compared to the controls. The regimes used in groups 1 and 5 appear to be the most effective therapeutic protocols.

of the available data, there does not seem to be a reasonable explanation for these findings. Experiments involving subcutaneous tumor implants were plagued by large variations in tumor volume within individual groups. It is possible, considering the low number of rats that were used in each group and the large variations that occur within individual groups, that this disparity was the result of random experimental variation.

An equally curious finding was that the group exhibiting the most dramatic decrease in tumor growth rate was Group 2, the group that received the lowest dose. Again, this was puzzling. In light of previous experimental data (Experiment B), the significance of this finding was questionable. None-the-less, this regime, along with the regime from Group 5 was chosen for future experiments (Experiment E.).

The remaining groups provide more reasonable data. The two control groups exhibited very similar growth curves. Group 3, which received an intermediate dose of NGF, exhibited an intermediate response. Groups 5 & 6 exhibited a dramatic decrease in tumor growth rate reminiscent of that identified in Experiment B. However, it did not appear that administering twice the dose (Group 6) over the same time frame (Group 5) provided added therapeutic benefit. As comparable results were obtained in Group 5 while using significantly less NGF, this schedule was adopted for future experiments.

In conclusion, a clearly superior treatment protocol was not identified. However, results indicate that; 1) NGF treatment is able to cause a decrease in the growth rate of subcutaneous T9 implants, 2) fewer tumors developed in the NGF treated groups compared to the untreated controls and 3) protocols 5 & 2 were appeared to be the most effective and should be considered for future experiments.

Experiments E: Stereotactically guided intracerebral implantation of T9 anaplastic glioma cells followed by NGF treatment directly at the implantation site

Following the success of the subcutaneous experiments involving T9 anaplastic glioma implants, Experiment E was undertaken to test the ability of NGF to diminish the growth rate of T9 implants in the brain. A number of obstacles needed to be overcome in order to successfully evaluate the response of an intracerebral tumor to NGF treatment. The principal problems that needed to be addressed were; 1) confinement of the implant to a localized area of the brain and 2) the development of a method whereby NGF treatment could be repeatedly administered directly at the intracerebral implantation site.

It was of prime importance to confine the tumor to a specific area of the brain so that differences in survival time would be a reflection of treatment and not of variations in tumor development. Several measures were taken to try and

accomplish this aim. The first was to place the implant deeper into a solid mass of parenchyma. Once this resolution was made, it became obvious that our previous implantation site, the parietal cortex, was inadequate. The parietal cortex was not an ideal place to implant the tumor because it did not offer sufficient parenchymal mass to entrap the tumor. In this region of the rat brain, there is a limited amount of cortex overlying the lateral ventricles. Furthermore, the implant cannot be placed deep into this region because it would then become established in the ventricular system or damage the underlying thalamic region. A large, solid area of cerebral parenchyma was needed that would tolerate an expansile mass. One of the few regions to fit this description is the striatum. The tumor could be seeded deep within the striatum to confine and contain the tumor to this location and lessen the likelihood of submeningeal dissemination.

Additional measures were taken to try and confine the tumor to a specific location, besides implanting the tumor deep within a solid mass of parenchyma. The volume of the implant was greatly diminished to try and prevent leakage from the needle tract. In addition, a very fine needle was used to decrease the size of the tract through which cells could escape. These measures were used in combination with a stereotaxic apparatus to insure precise implant placement in each animal.

With measures taken to confine the tumor to a specific location, the second problem could be addressed. A system was needed whereby repeated treatments could be delivered to the intracerebral implantation site. This would avoid systemic dilution of the administered dose of NGF and by-pass the necessity to traverse the blood-brain-barrier. The development of such a system represented a significant obstacle. To repeatedly place the animal in a stereotaxic apparatus in order to administer the treatment directly at the implantation site would have been far too traumatic. Such a procedure would require repeated anesthetic episodes and repeated placement of the animal in the stereotaxic apparatus. To restrain a rat in the stereotaxic apparatus is not a benign procedure. The tympanic membrane is ruptured when the animal is placed in the apparatus and repeated procedures could result in middle and inner ear infections. More importantly, in order to accurately locate a specific area of the brain with a stereotaxic apparatus, it must be calibrated from a landmark, either lambda or bregma, present on the surface of the animals skull. This would necessitate repeated incisions through the skin to locate one of these landmarks. This process was considered too traumatic. An alternative would be to place a guide through the skull to provide a access route for the treatments. Such a procedure would offer the following advantages: 1) it would eliminate the repeated surgery and associated trauma necessary with stereotaxic

techniques 2) animals would not need to be completely anesthetized to perform the treatments and 3) the needle guide would provide a reliable access route to the implantation site which would insure that the treatment was administered at the appropriate location.

The implantation of a needle guide was determined to be the method that most adequately fulfilled our needs. This procedure did have one significant drawback. Several experiments had demonstrated that 35 ug NGF administered once a day for five consecutive days was an effective protocol. However, this protocol seemed too traumatic to be used with this system. Animals would need to be sedated daily for five consecutive days and submit to a needle being passed through their brain. For the welfare of the animal, it was decided to spread the treatments out over a longer period of time.

Materials and Methods

90-110g Fischer 344 rats were anesthetized with a Rompun/Ketamine mixture administered IP (55 parts Ketamine 100mg/ml + 45 parts Rompun 20 mg/ml) at a dose of 0.1 mls/100g. The animal was positioned in a stereotaxic apparatus (Kopf Model 900 Small Animal Stereotaxic apparatus). The animals head was shaved and swabbed with alcohol. A 2 cm midline incision was made starting 0.5 cm posterior to the orbit and extending slightly beyond the nuchal crest. Once the skin had been reflected, the scalpel blade was again

passed over the midline surface of the skull to free any remaining subcutaneous tissue which was then reflected. A mark was made on the surface of the skull at the coordinates of bregma and 3.5 mm to the left of the mid-sagittal suture. A 0.8 mm hole was drilled at this point. Three addition holes were drilled through the skull, one 5 mm cranial and slightly medial to the first and two 5 mm caudal. These three holes formed a triangle centered around the first hole. Three #00 x 1/8" self-taping stainless steel screws (TX00-2 Small Parts Inc, Miami FL) were placed in the peripheral holes leaving 3/4 of the screw exposed. A 10 mm section of 23 gauge thin walled hypodermic tubing was stereotaxically positioned in the central hole to a depth of 1mm below the surface of the skull. This stilet was cemented in place with dental acrylic. The dental acrylic formed a cap on the surface of the skull with the three peripheral screws acting as anchors. Once the dental acrylic was dry, a 27 gauge needle was passed through the stilet and 10^4 T9 cells (2 x 10^6 cells/ml) in 5 ul DMEM-O was implanted 4.5 mm deep into the left striatum. The stilet was then capped and the caudal portion of the incision closed with 4-0 Ethylon suture. Animals were housed in individual cages.

In order to administer the treatments, rats were sedated with 0.05 mls of an acepromazine/ketamine mixture IP (2 parts ketamine (Ketaset, Bristol 100mg/ml) + 8 parts acepromazine (PromAce, Fort Dodge 10mg/ml)). Treatments were administered

through the needle guide so that the solutions could be injected directly at the implantation site. The nine control animals received 20 ul of the final dialysate solution on days 8, 11, 14 and 17 post-implantation. The eight animals in the single dose NGF treatment group received a single injection of 35 ug NGF in 20 ul 0.9% saline on day 8. The eight animals in the multiple dose NGF treatment group received 35 ug NGF on days 8, 11, 14 and 17 post-implantation for a total dose of 140 ug NGF. NGF activity was assessed prior to the experiments and once treatment had been completed using an *in vitro* bioassay. The sutures were removed at the time of the first treatments.

Animals were observed three times per day and euthanized with CO_2 once they began to exhibit progressive neurologic signs. The experimental subjects were evaluated in terms of survival time, and tumor volume at the time of euthanasia divided by survival time. Routine histologic sections of the intracerebral tumors were also evaluated. The brain was sectioned at the level of the implantation site, and 4.0 mm cranial and caudal to the implantation site. The brain stem and cerebellum were sectioned sagittally 2 mm from the median plane.

Results

In one respect, these experiments were quite successful. Each animal developed a tumor at the implantation site and,

in all but one animal, the neoplastic proliferation was confined solely to the implantation site in the striatum. Therefore, it would appear that measures taken to confine these tumors to the implantation site were very effective. The method whereby NGF treatment was repeatedly administered to the implantation site also appeared to be very successful. The rats tolerated the procedure quite well. Complications were not encountered. Rats did not develop meningoencephalitis or surface infections associated with the presence of the needle guide. Self-inflicted trauma associated with the presence of the needle guide was not a problem.

However, as table 2.3 illustrates, NGF treatment did not result in a statistically significant difference in either average survival time or tumor size at euthanasia divided by survival time. There was essentially no difference between the various groups in terms of survival time or in the measure of tumor growth rate.

Discussion

Stereotactic implantation of a needle guide proved to be a very useful technique. The procedure allowed for the precise placement of the implant into the striatum followed by direct treatment of this area. Remarkably, complications associated with the implantation and maintenance of the needle guide were nonexistent.

Table 2.3: The effect of NGF treatment on stereotactically guided intracerebral T9 implants.

	Average Survival Time	Tumor volume/ survival time
[a]Control:	27.37 days	6.84 mm^3/day
[b]Single Dose NGF: (35 ug total dose)	27.0 days	7.30 mm^3/day
[b]Multiple doses NGF: (140 ug total dose)	27.37 days	6.38 mm^3/day

[a] N = 9
[b] N = 8

However, neither the single dose- or multiple dose-NGF treatments resulted in a decrease in tumor growth rate or an increase in survival time. This was very puzzling considering the success of experiments involving subcutaneous implants. A number of factors may have contributed to this failure. It is quite possible that the change in therapeutic regime may have diminished the efficacy of the treatment. It was considered too traumatic to treat these animals daily at an intracerebral location and we were limited by the amount of NGF that could be administered during each intracerebral treatment. As such, treatments were spread out over a longer period of time and a lower total dose was administered. It is quite possible that changes in the total dose and dosage schedule may have decreased efficacy. There is also a distinct possibility that serum leakage following each NGF treatment may have diminished the effectiveness of the NGF.

The preceding paper describes the adverse effects associated with the present of serum when administering NGF treatment. In the rats, hemorrhage associated with subcutaneous NGF treatments was rare and only occurred when the animal moved violently while the needle was positioned in the subcutis. However, a completely different set of circumstances was encountered in the brain. The tumor was implanted deep into the striatum. As such, the meninges and several millimeters of cerebral parenchyma had to be traversed in order to reach the implantation site. This greatly

increased the likelihood of serum leakage and/or hemorrhage at the treatment site which would diminish the amount of NGF available to induce reverse transformation of the neoplastic cells. It should be noted that on a number of occasions following treatment a serosanguinous fluid welled up through the needle guide indicating that significant hemorrhage had occurred. Lastly, we may have been too efficient in confining the tumors to a localized area in the brain. Even though fewer cells were implanted in this experiment, survival time was decreased compared to Experiment B. Tumors produced by the implantation of T9 anaplastic glioma cells are aggressive and grow very rapidly. Because the growth of these T9 anaplastic glioma implants is so rapid, this model may not truly reflect the ability of NGF to diminish the growth rate of slowly expanding naturally occurring CNS tumors.

These technically difficult and unrewarding studies were abandoned in favor of subcutaneous experiments. The subcutaneous studies allowed for better control of the treatment procedures and the ability to continuously monitor tumor growth. Results from subcutaneous experiments provide the data on which the preceding paper was based.

Intracerebral implantation of clone 16

Despite the failure of the previous experiment, thoughts of additional intracerebral studies were not completely abandoned. Following the success of subcutaneous experiments

involving the neurinoma clone, clone 16, intracerebral experiments utilizing this cell lines were contemplated. However, before intracerebral studies could be considered, information on the consistency of tumor development and the growth rate of intracerebral clone 16 implants needed to be obtained.

Materials and Methods

A needle guide was implanted for this study using a procedure similar to that described for the previous experiment, Experiment E. Four animals received implants. A suspension of 10^4 clone 16 cells (passage 93) in 5 ul DMEM-0 (2 x 10^6 cells/ml) was implanted into the left striatum via the needle guide. Animals were euthanized at 30, 60, 97, and 128 days post-implantation. The brain was sectioned at the level of the needle guide and both faces of this section were examined for evidence of tumor. In addition, sections 4.0 mm cranial and caudal to the implantation site, and sagittal brain stem and cerebellum sections cut 2 mm from the median plane were evaluated.

Results

None of the experimental subjects developed progressive neurologic signs during the course of this study. Tumors were not apparent on gross inspection of brain sections at the time of necropsy. On histologic examination the needle track was

successfully identified in each animal indicating that sections had been taken at the appropriate level. However, a neoplastic proliferation was not identified in any of the sections.

Discussion

Though clone 16 implants grew readily in the subcutaneous tissue of Fischer rats, none of the rats receiving intracerebral implants developed tumors. At necropsy, though the needle tract was successfully identified histologically, these was no evidence of tumor formation either at the implantation site or elsewhere in the brain. Tumors were not apparent at any time period. There was no evidence that neoplastic cells had survived or proliferated at the implantation site in the brains of these animals.

It has been observed that neurinomas, tumors of probable Schwann cell origin, may grow directly up to the brain but will not infiltrate the CNS tissue. Apparently, the environment of the brain is not conducive to the survival and growth of tumors of Schwann cell origin. This appears to be true with clone 16, a tumor of probable Schwann cell origin. Though these tumors grew vigorously in the subcutaneous tissue, they did not grow in the environs of the brain. Therefore, further consideration of the use of clone 16 implants for intracerebral studies was abandoned.

Experiments involving the implantation of two human tumor cell lines into athymic nude mice

The preceding article mentions results from experiments in which human tumor cell lines were implanted into irradiated nude mice. The article states that "In contrast to the rodent tumor cell lines, only a minimal response to NGF (35 ug NGF SID for 6 days) was observed following treatment of these tumors (U251 and D54) in nude mice. In each case, NGF treatment resulted in a slight decrease in growth rate (data not shown). However, the decrease was not nearly as dramatic as with the rodent tumor lines and was not statistically significant." The results of these experiments are illustrated in this appendix (Figures 2.6 and 2.7). The "Materials and Methods" and "Discussion" for these results are contained in the preceding article. These graphs were included to clarify the statement that "NGF treatment resulted in a slight decrease in growth rate."

Figure 2.6: The effect of NGF treatment on the growth rate of subcutaneous U251 glioblastoma multiforme implants

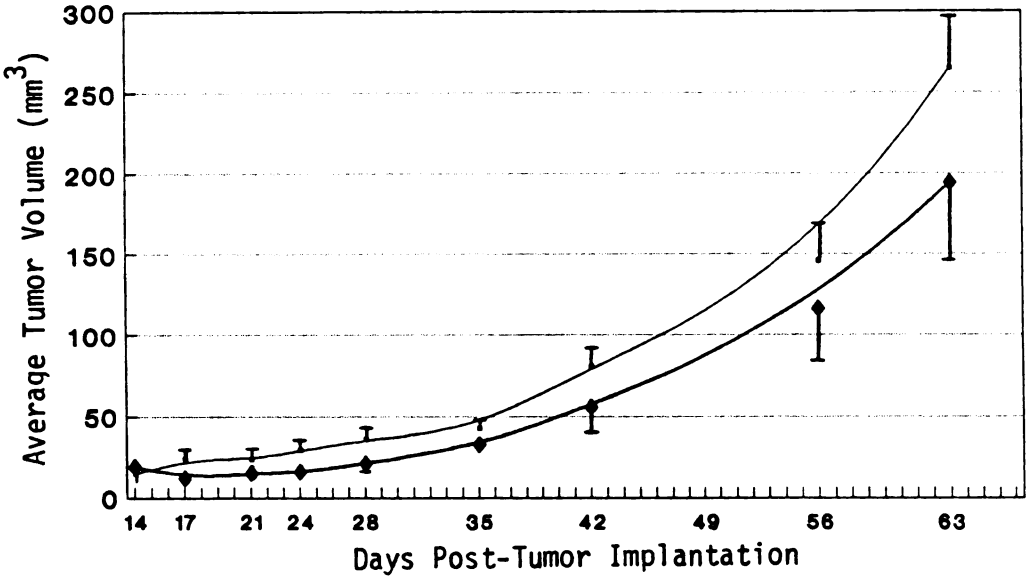


Figure 2.6 illustrates the effect of NGF treatment on the growth rate of U251 implants in irradiated nude mice. The growth rate of U251 implants was decreased slightly in NGF-treated animals (•) (35 ug SID for 6 days) when compared to the tumor growth rate in untreated control mice (•). This difference was not statistically significant.

Figure 2.7: The effect of NGF treatment on the growth rate of subcutaneous D54 mixed glioma implants

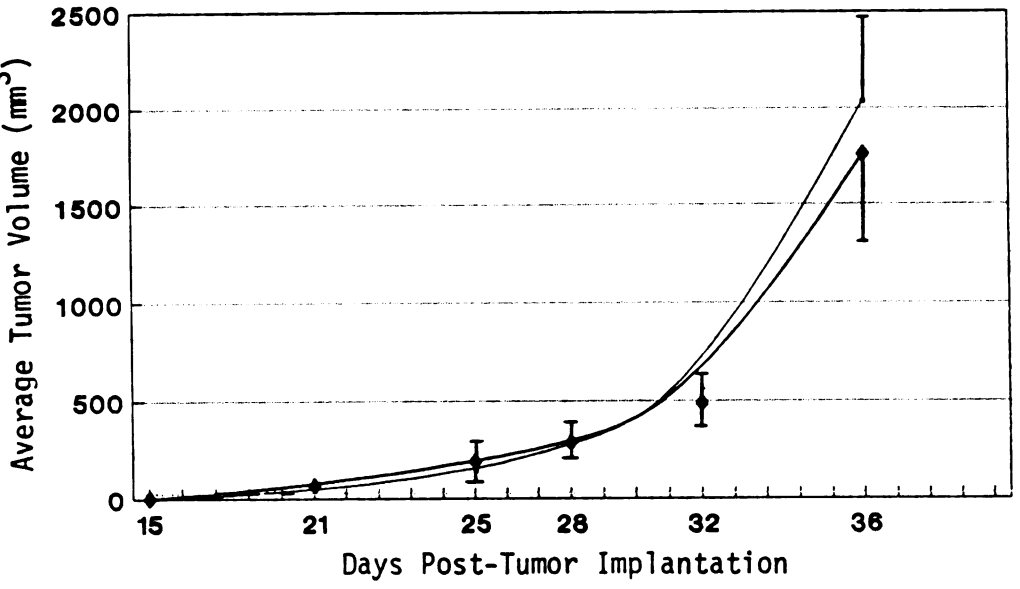


Figure 2.7 illustrates the effect of NGF treatment on the growth rate of D54 implants in irradiated nude mice. When the growth rate of D54 implants in NGF-treated (•) and untreated (•) mice are compared, it is apparent that the difference between the two groups is minimal and not statistically significant. Though it may be difficult to appreciate from the graph, during the initial measurement period, tumors in the NGF treatment group were actually significantly larger ($P < 0.05$) than tumors from the untreated control. On day 21 post-implantation, tumors in the NGF-treated group were twice the size of tumors in the untreated control group. As the experiment progressed this trend gradually reversed, until at the time of necropsy, tumors in the NGF-treated group were only 86% the size of tumors in the untreated control group.

Conclusions; Chapter 3 Appendix

This appendix provides addition information on some of the *in vivo* techniques not described in the preceding paper. It also discusses some of the pitfalls associated with these types of experiments. Though a number of problems were encountered during the course of the *in vivo* studies, the dramatic decrease in the growth rate of both T9 anaplastic glioma and clone 16 neurinoma implants in response to NGF indicates that the growth factor possesses significant therapeutic potential. Results from chapters 1 and 2 provide persuasive evidence that NGF may indeed prove useful for the treatment of tumors of neurogenic origin.

CONCLUSIONS AND FUTURE STUDIES

A pertinent question to ask at this point would be, "what do I perceive is the potential of this type of therapy and in what direction should research in this area proceed?"

There are a number of valid approaches to cancer therapy. Surgical removal, when an option, is without question the most effective. Destruction of neoplastic cells through the use of radiation or chemotherapy has also proven effective. However, radiation and chemotherapy are generally plagued by fairly severe adverse side effects, and the outcome of therapy is generally remission and not complete cure. Therefore, there is a definite need for the development of alternative modes of therapy, especially in the area of brain tumor therapy where surgical removal is often not an option.

The aforementioned forms of therapy rely on the removal or destruction of neoplastic cells. These approaches, though certainly effective, do not address the fundamental processes involved in neoplastic transformation. They rely on annihilation of the neoplastic cell. The proximate cause of cancer is considered to be altered or aberrant expression of normal cellular genes important in growth and development. An equally valid approach to cancer therapy would be to confront the disease directly at the pathways responsible for neoplastic transformation. If pathways essential for the maintenance of the transformed phenotype could be persistently

suppressed, the neoplastic behavior of transformed cells would be effectively controlled. This is the principle upon which reverse transformation is based.

Reverse transforming agents stimulate neoplastic cells to differentiate to the point where they no longer exhibit neoplastic behavior. In this process the expression of pathways essential for maintenance of the transformed phenotype become suppressed. As we learn more about the mechanisms involved in neoplastic transformation and about the processes involved in differentiation, I believe we will identify points at which these two processes intersect. It is at these points that reverse transforming agents may be able to intervene in a therapeutically beneficial manner.

Reverse transformation is a conceptually sound approach that has two additional advantages. Because natural differentiating agents are employed, there is the potential for diminished systemic toxicity, especially if the agent can be administered locally and confined largely to the region of the tumor. These agents also have the potential for increased specificity over many types of chemotherapeutic agents because only those cells possessing receptors and functional response pathways will be affected.

I believe the concept of reverse transformation is valid. Indeed, rather than annihilating the neoplastic cells because we are unable to regulate neoplastic behavior, reverse transformation attempts to control neoplastic behavior by

suppressing pathways responsible for maintenance of the transformed phenotype. The more I contemplate this approach, the more I am convinced that this is a valid strategy. I also believe that NGF possess significant reverse transforming potential. The preceding chapters chronicle the basis for this belief.

If I were to continue to pursue this line of research, there are three areas that I would be particularly interested in exploring. First, there is a need to further characterize the persistent nature of the NGF-induced response. Because reverse transformation does not result in the direct elimination of neoplastic cells, the effects must be highly persistent. Data presented in Chapter 2 demonstrates the persistent nature of the NGF induced effects in vitro. However, these experiments were conducted over a relatively short period of time, considering the goals of cancer therapy. Because persistence is such a vital component of reverse transformation, the fate of neoplastic cells treated with NGF needs to be critically evaluated. Studies must demonstrate that, at least in a subpopulation of neoplastic cells, the effects of NGF are highly persistent if not permanent. Because tumors are composed of a heterogeneous population of cells, the various subpopulations will have to be separated and evaluated individually for persistence. If it can be demonstrated that the reverse transforming agent can persistently or permanently suppress the growth of a

subpopulation of cells within the neoplasm, then a therapeutic advantage will have been gained.

Second, it would be beneficial to conclusively establish that the reverse transforming agent has suppressed pathways essential for maintenance of the transformed phenotype. Evidence presented in the preceding chapters clearly demonstrates that NGF is capable of suppressing the growth of neoplastic cells both *in vitro* and *in vivo*. The next step would be to identify the mechanisms whereby this is achieved. This could be accomplished by using cell lines in which the defects leading to neoplastic transformation have been well characterized or by transfecting cells with known oncogenes. Demonstration that treatment has suppressed known pathways essential for transformation would provide conclusive support for this approach.

Finally, it would be interesting to see if a synergistic effect could be achieved by combining NGF with other known differentiating agents, such as TGF-beta and beta-carotene. Just as combinations of chemotherapeutic agents are significantly more effective then any single agent alone, combinations of reverse transforming agents may also prove more effective.

I feel strongly that the concept of reverse transformation is a valid approach to cancer therapy and that NGF possesses significant reverse transforming potential. This area of research has been interesting and rewarding. The

ability of NGF to drastically diminish the growth rate of two of the neurogenic tumor cell lines when implanted into syngeneic animals demonstrates potential therapeutic promise that requires further investigation.

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