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Proliferation, Morphologic and Functional Characteristics of Alkylnitrosourea-Induced Astrocytoma Cells and Changes Induced by Nerve Growth Factor

By

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ABSTRACT

Proliferation, Morphologic and Functional Characteristics of Alkylnitrosourea-Induced Astrocytoma Cells and Changes Inducible by Nerve Growth Factor

By

William Russell Hare, Jr.

Nerve growth factor (NGF) is a potent differentiation promoter of astrocytes. In response to treatment with NGF, astrocyte morphology changes and cells take on the end stage appearance of protoplasmic and fibrous astrocytes. Anaplastic astrocytoma cells also have been reported to respond with changes suggesting promotion of morphologic differentiation. In addition, astrocytoma cells have been reported to respond to NGF treatment by a reduction in growth. NGF has been shown to reduce the size of tumors *in vivo* and to control the growth of tumor cells *in vitro* . Because of these reverse transforming characteristics, NGF has been suggested as a treatment for astrocytomas. However, aside from these observations on tumors and tumor cells, little is known of the molecular mechanisms involved in NGF's action. There also is very little information available, outside of morphologic studies, on the effects of NGF on normal astrocytes. Therefore, the purpose of this dissertation was to examine and compare the action of NGF on neonatal astrocytes and anaplastic astrocytoma cells *in vitro*, and with respect to morphology, function and proliferation. Morphology was studied by determining the effects of NGF on cellular anatomy, characterized by formation of cytoplasmic processes and intermediate filaments. These areas

were investigated by using light-microscopy as well as immunofluoresence. Function was studied by determining the effects of NGF on the uptake of glutamate (GLU) and γ-aminobutyric acid (GABA), the major excitatory and inhibitory amino acid neurotransmitters, respectively. This area was investigated by use of ^{3}H-GLU and ^{3}H-GABA and scintillation analysis. Proliferation was studied by determining NGF induced changes in the cell cycle of these two cell populations. This was investigated by use of acridine orange flow cytometry.

NGF treatment of cell cultures in the presence of glutamate resulted in morphologic differentiation of astrocytoma cells from monotonous spindle shaped cells to cells resembling protoplasmic and fibrous astrocytes. NGF increased the intensity of glial fibrillary acidic protein with perinuclear, nuclear and nucleolar distributions. Amino acid neurotransmitter uptake was not adversely affected by NGF treatment. NGF was also found to significantly affect proliferation potential of astrocytoma cells by inducing a quiescent non-cycling subpopulation of cells.

This dissertation is dedicated to the memory of my wife **Jeannette C. Hare**.

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Introduction

Primary brain tumors are comprised of an array of diverse neoplastic cell types, each with its own characteristic morphology and growth pattern. The most prominent of these brain tumors are those derived from neuroectodermal origin. Cells of neuroectodermal origin differentiate into four morphological and functionally distinct cell types. They are neurons, astrocytes, oligodendrocytes and ependymal cells. Bailey and Cushing were the first to compare tumor cells with developmental stages of normal cells (Bailey and Cushing, 1926). Their classification of brain tumor cells resulted in an orderly classification of what Virchow had earlier categorized inclusively and termed glioma (Virchow, 1846, 1854). Although the Bailey and Cushing classification has been subjected to a number of criticisms and modifications by pathologists through the years, it has remained the major basis for pathological diagnosis and classification of intracranial tumors .

As a result of this classification scheme, gliomas were found to constitute half of all intracranial tumors. In addition, this classification system introduced the thought that tumor cells were actually aberrant cells of one of the developmental stages of normal cells. Astrocytomas were thought to be represented by aberrant glioblasts or spongioblasts and therefore were named and classified as glioblastoma and spongioblastoma. Histologically cells were also classified by their degree of anaplasia, hence differentiated and anaplastic astrocytomas became common terminology for astrocytic tumors.

Chemical Carcinogenesis

As a result of the classification system introduced by Bailey and Cushing, comparative histology became the norm for giving a more specific identity to tumors. However, there was still a lack of information on the genesis of brain tumors. It wasn't until the 1930's and the advent of experimentally induced brain tumors in laboratory animals that greater understanding of the process of neoplastic transformation and carcinogenesis in general began to unfold. The first reports on experimentally produced brain tumors were the production of brain tumors following intracerebral application of polycyclic aromatic hydrocarbons (Seligman and Shear, 1939). Since then, many chemical compounds have been reported to selectively induce tumors in the nervous system (Kleihues *et al*, 1976; Bigner and Swenberg, 1977). These neuro-oncogenic agents have also been reported to selectively induce tumors of the nervous system through various routes of administration.

Epidemiological studies in more recent years have provided convincing evidence that environmental factors including chemicals do play a major role in the causation of brain tumors (Moss, 1985). These studies have also brought forth some optimism, since they imply that the incidence of brain tumors might be reduced by controlling and cleaning up our environment.

Carcinogenesis is a pathological process that proceeds through defined stages of initiation, promotion and progression. It is very likely that the transition from one stage to another is under the influence of separate environmental or endogenous factors. Therefore, naturally occurring tumors and experimentally induced tumors may share similar mechanisms of

causation and development. These mechanisms of carcinogenesis can be studied by examining the biochemical reactions involved in the development of experimental brain tumors while at the same time considering the importance of genetic variations.

Several types of chemicals have been found to initiate cancer in animal models due to the generation of highly reactive intermediates which bind covalently with the nucleic acids of the target cell nucleus. This covalent binding to the DNA of cells is an essential step in the initiation of neoplastic transformation. The changes in DNA structure which result, in turn, interfere with normal DNA function during replication and transcription. These changes in DNA function have been found to involve molecular pathologic processes, which interfere with normal gene expression. These molecular processes include oncogene activation, inhibitory gene suppression, DNA amplification, gene transposition, chromosome translocations and gene deletions.

Alkylnitrosoureas and Experimental Brain Tumors

An ideal experimental brain tumor model can be produced by alkylnitrosourea compounds in CD or Fischer rats (Koestner *et al*, 1971; Schmidek *et al*, 1970). Alkylnitrosoureas at specific dose dependent concentrations are found to produce tumors of the brain and nervous system in more than 90% of the offspring of pregnant rats following prenatal administration (Druckery *et al*, 1965). The neurotropic action of these compounds is believed to be due primarily to the action of their urea radical, which facilitates their diffusion into the CNS (Laerum *et al*, 1978). These compounds are small in size, lipid soluble and extremely reactive. Like other alkylating carcinogenic agents, their action is mutagenic and results from the

alkylation of specific DNA bases. The carcinogenic action of both methylnitrosourea (MNU) and ethylnitrosourea (ENU) in turn has been linked, by some, to a defect in the excision repair mechanism of these alkylated bases from brain DNA. Nitrosoureas are found to cause alkylation at N^7- and O^6- positions of guanine (Singer, 1975). The production of N^7-alkyl guanine adducts are usually quickly and efficiently eliminated by chemical, non-enzymatic depurination. This action in large part is due to the lability of the glycosyl bonds in the DNA chain. Thus, depurination follows first order kinetics with a half-life of approximately 155 hours for N^7-alkylguanosine. In addition to spontaneous glycosyl bond cleavage, alkylation products may also be enzymatically removed by the action of specific glycosylase enzymes (Kleihues and Margison, 1974; Lindahl, 1976; Bigden *et al* 1981).

Inability or failure of a cell to eliminate the O^6-alkylation of guanine has been suggested to be the molecular lesion responsible for the carcinogenic action of the alkylnitrosoureas in rats (Goth and Rajewsky, 1974). This specific lesion results in the mis-pairing of guanine with thymine instead of cytosine. This anomalous base-pairing results in a somewhat stable but defective conformation to the DNA molecule (Goth and Rajewsky, 1974; Margison and Kleihues, 1975). However, even though O^6-methylguanine has been shown to be the specific lesion linking MNU to its carcinogenic activity, species and strain-related differences have not been paralleled by differences in the excision repair capacity of O^6-methylguanine adducts (Kleihues *et al*, 1979).

The gerbil has been shown to have less capacity to repair O^6-methylguanine than the rat. Yet, the gerbil is not susceptible to the neuro-oncogenic effects of MNU (Swenberg, 1986). However, MNU does induce a benign melanoma of the skin in the gerbil. Variations in the effects of

chemical carcinogens in different species such as the gerbil illustrate the influence of genetics on the process of carcinogenesis.

Astrocytes and Intermediate Filaments

Astrocytes, as their name implies, are star-shaped cells. End-stage adult-differentiated astrocytes are represented by two morphologic types, protoplasmic and fibrous. Protoplasmic astrocytes are characterized by having an epithelioid morphology while fibrous astrocytes are characterized by having a stellate morphology. Therefore, their morphologic distinction is the result of their cell process development and ultimate shape. In normal brain, protoplasmic astrocytes are generally confined to areas of gray matter which constitute the cerebral and cerebellar cortices and basal ganglia while fibrous astrocytes inhabit the white matter (Leeson and Leeson, 1981).

Protoplasmic and fibrous astrocytes can also be distinguished by the presence, location and content of intermediate-sized intracellular filaments. These intermediate filaments (IFs) measure 7-11 *n*m in diameter and form compact bundles in fibrous astrocytes and scantier arrays in protoplasmic astrocytes (Russell and Rubenstein, 1989). IFs of differentiated astrocytes have been reported to be formed by polymerization of a biochemically and immunologically distinct class of proteins, the glial fibrillary acidic protein (GFAP). Since the discovery of IF's in the 1970's and their separation into distinct cell-specific classes, they have been used for cell identification. Therefore, GFAP immunofluorescence was used for the identification of astrocytes. Tumors now could be classified not only by morphology but by use of a cell-specific marker for GFAP.

Another IF protein, vimentin, has also been found in various cells of mesenchymal origin including astrocytes. In some mammalian species,

vimentin has been found to constitute the major IF protein of immature developing astrocytes (Bignami *et al*, 1980,1982; Bignami and Dahl, 1974). However , in adult, differentiated astrocytes, vimentin has been found to be a minor component which co-exists with GFAP (Bjorklund *et al*, 1984).

Under pathological influences, protoplasmic astrocytes have been reported to be rapidly and permanently converted to fibrous astrocytes (Russell and Rubenstein, 1989). This process of gliosis is probably the most frequent and least specific of all the cellular events correlating with neuropathology and is easily identifiable by the immediate increase in GFAP expression.

Since the discovery of IFs they have been assumed to be components of the cytoskeletal system and partly responsible for cell shape (Ishikawa *et al*, 1968; Lazarides, 1980). However, recent experiments seem to indicate that IFs either play a more subtle role in the cytoskeletal system or may only represent a storage or transportation form of their functional subunit-proteins. IFs have also been demonstrated to be nucleic acid-binding proteins and susceptible to limited processing by Ca^{++} activated neutral thiol proteases (Nelson and Traub, 1981; Bigbee *et al*, 1983). They have been further characterized by their preferential binding to 18s ribosomal RNA and single stranded DNA. However, the binding potential of IFs has not only been determined by structural conformation but by base composition. IF binding potential has been shown to be greatest by guanine (Traub and Vorgias, 1983; Traub, 1985). Therefore, IF subunit-proteins have been suggested to carry out a role in membrane transduction for the transformation of extracellular signals to intracellular targets by acting as second messengers.

Growth Factors and Differentiation Promoters

Differentiation promoters have been known to play important roles in normal neural development. Among these differentiation promoters are numerous growth factors and hormones. Nerve growth factor (NGF) is a potent differentiation promoter. It is a polypeptide that is required for the maturation and maintenance of sympathetic and sensory neurons as well as certain cholinergic neurons (Levi-Montalcini and Angeletti, 1968; Hefti, 1986; Williams *et al*, 1986; Levi-Montalcini, 1987; Greene and Shooter, 1980; Yankner and Shooter, 1982; Bradshaw, 1978). NGF's mechanisms of action have been studied largely by use of the rat pheochromocytoma, PC12, cell line. NGF's promotion of neuronal differentiation has been well studied by using the PC12 cell model. NGF has been found to be basically a non-mitogenic growth factor (Cohen *et al* 1954). It has only been shown to be mitogenic to certain established cell lines or during specific periods of development (Lillen and Claude, 1985; Burstein and Greene, 1982). Research involving NGF has focused heavily on the mechanisms that control cell development and differentiation.

Previous studies have indicated that NGF controls the movement of Na^+ and K^+ across the cell membrane through regulation of the Na^+, K^+ pump (Varon and Skaper, 1980; 1983). The control of ionic equilibrium has further been shown to be fundamental to changes in cell proliferation, extension and elongation of cytoplasmic processes as well as general cell maintenance and repair (Rozengurt and Mendoza, 1980; Jaffe and Nuccitelli, 1977; Becker, 1981). Therefore, ionic control by NGF may be the key to its action on cell differentiation (Varon and Adler, 1981; Varon and Skaper, 1980,1983).

Cell Surface Receptors

Growth factors and hormones share a common dependency on surface membrane receptors to initiate their actions on responsive cells (James and Bradshaw, 1984). In addition, molecular characterization of receptors for both growth factors and hormones has revealed many similarities in their structural and functional characteristics. There are two broad classes of growth factor receptors, those with and without intracellular tyrosine kinase activity. Most of these receptors are divided into three domains: extracellular, transmembrane and intracellular. The receptors for insulin and insulin-like growth factor, for example, are composed of four polypeptides. These polypeptides are symmetrical dimers of 2 α- and 2 β-chains. Each chain in turn is derived from a single precursor polypeptide. This precursor expresses the same three domains as other growth factor receptors (Ullrich *et al*, 1985; Ebina *et al*, 1985). These receptor proteins are also glycosylated during processing by O- and N-linked glycosylation. In addition they are each rich in disulfide bonds which are often found to be clustered into subdomains which strongly influence their tertiary structure. Both glycosylation and tertiary structure play important roles in ligand binding and subsequent activation.

Signal Transduction

The molecular responses that result from growth factor-receptor interaction are for the most part poorly understood. However, they are capable of inducing transmembrane signals, which are amplified by a variety of second messengers. Membrane transduction results in immediate and delayed response. Changes in gene expression are an example of a delayed response and are thought to be the result of growth factors or altered growth factors interfering with translation or transcription processes (James and

Bradshaw, 1984). Immediate responses are thought to result from changes in ionic regulation by the membrane or from the direct action of second messengers like Ca^{++} and cyclic-AMP.

Cyclic-AMP is a second messenger which is the product of adenyl cyclase activation. Adenyl cyclase activation results from the binding of growth factors to G (GTP) binding proteins, which are enzyme-linked proteins. Other second messengers include inositol trisphosphate (IP3) and diacylglycerol (DAG) along with Ca^{++} and phosphorylated proteins. Phosphorylation takes place as a result of kinase activation. These second messengers are all generated by the activation of phospholipase enzymes. Therefore, the combined activation of phospholipase and adenyl cyclase could also be responsible for many of the immediate cellular responses generated by growth factors.

Endocytosis

Another consequence of growth factor-receptor complex formation is induction of endocytosis. The process of endocytosis is thought to be responsible for the inactivation of many growth factor- receptor complexes. It also appears possible that this process is necessary for delayed and prolonged forms of signal transduction. Growth factors can be transported by endocytic vesicles, either intact or in an altered form due to processing by enzymatic action, to the nuclear membrane. Nuclear interaction is characterized by changes in gene expression which result from the interaction of these growth factor messengers.

IFs also share a role in receptor-mediated endocytosis. IFs and microtubules undergo a redistribution shortly after cap formation of the growth factor-receptor complex. These filaments accumulate in the uropod

in a parallel fashion to the nucleus-cap axis (Zucker-Franklin *et al*, 1979; Butman *et al*, 1980, 1981; Dellagi and Brouet, 1982). In resting cells, IFs have been reported to occupy a waiting position in the perinuclear region. When cells become stimulated IFs radiate out into a more open fibrillar network to make contact with endocytic vesicles. Therefore, IFs appear to play an important role in regulation of growth factor-receptor complex inactivation and may be responsible for their subsequent activity as transducers of delayed and prolonged membrane stimulated information systems.

Oncogene Involvement

The transformation of cells from a normal physiologic state to a neoplastic state has been shown in numerous instances to be accompanied by increased oncogene expression (Fujimoto *et al.*, 1988; Thompson *et al.*, 1986; Gordon, 1985). This activity usually is thought to relate to the initiation step in the process of neoplastic transformation. Specific mutations in brain tumors have been shown to involve the expression of various oncogenes at different locations of the cellular genome. These oncogene products disrupt normal membrane transduction through their molecular mimicry of specific components of the growth factor-receptor interaction. However, oncogene amplification may not always be an initiating event in carcinogenesis. Enhanced expression of c-*myc* and N-*myc* has been shown to occur during tumor progression (Little *et al*, 1983; Schwab *et al*, 1983). In addition, *myc* activation has been shown to be tightly coupled to growth stimulation of quiescent cells and, therefore, may be related somehow to the entry of cells into and through the G1 phase of the cell cycle (Campisi *et al*, 1984; Kelly *et al*, 1983). It has also been suggested that the *myc* proteins may regulate the expression of other gene products by altering their relationship to the nuclear

matrix or by directly interacting with regulatory sequences (Eisenman *et al*, 1985). This latter possibility is reflected by the affinity of *myc* proteins for DNA. This, as well as other information on nuclear acting oncogene products, suggests that a definite relationship exists between nuclear structure and transformation. However, amplified oncogenes have also been shown to become transcriptionally silent following induction of tumor cell differentiation (Thiele *et al*, 1985; Westin *et al*, 1982).

Astrocyte Function

Until the last few years, very little was known of the function of astrocytes. They were simply thought to serve as a means of physical support for CNS neurons. It had been established that they were involved in the production of scar tissue following mechanical brain injury and, once established, remained permanently. But, this rather static view of glial cells relegated them to only a passive and supportive role, suggesting that their loss would be of no great consequence to a normal functioning nervous system. However, glial cells are no longer thought to function passively. There has been a dramatic change in the way glial cells are thought to function and in the role they play in the homeostasis of the CNS. Numerous reviews are now available attesting to the active nature and to the importance of glial function to a normal functioning brain (Hertz, 1978; Schoffeniels *et al*, 1978; Varon and Somjen, 1979; Stewart and Rosenberg, 1979). Glial cells are now depicted as modulators of neuronal function. They have been shown to act as cellular buffers in the establishment and maintenance of the blood brain barrier which regulates the extracellular environment as well as to control electrolyte abnormalities and the levels of amino acid neurotransmitters through active uptake systems.

The presence of large swollen astrocytes in a number of instances of brain edema was interpreted by many and suggested by others to mean that astrocytes play a functional role in the regulation of water and electrolytes (Gerschenfield *et al*, 1959; Katzman, 1961; Tower, 1966; Wasterlain and Torack, 1968; Hirano, 1969). It was soon discovered that astrocytes regulated extracellular K^+ (Kuffler and Nicholls, 1966; Orkland *et al*, 1966; Haljamae and Hamberger, 1971; Henn *et al*, 1972; Kimelberg, 1974; Bourke *et al*, 1975; Hertz, 1978).

Astrocyte involvement in ammonia metabolism may be one of their more important functions . Ammonia is constantly produced in the brain and increases with increased neuronal activity (Richter and Dawson, 1948; Tsukada, 1971; Quastel, 1974, 1979). In addition, the brain has been reported to take up ammonia when ammonia levels increase in blood (Webster and Gabuzda, 1958; Hindfelt, 1975; Lockwood *et al*, 1979). The role of astrocytes in regulating ammonia concentrations is directly related to capillary density and maintenance of the blood brain barrier (Phelps *et al*, 1977). Reactive and degenerative changes in astrocytes and the production of Alzheimer type II astrocytes in conditions of hyperammonemic states has further substantiated the proposed role of astrocytes in ammonia detoxification (Zamora *et al*, 1973; Norenberg, 1977).

Astrocytes also play an important role in the uptake and metabolism of amino acid neurotransmitters. The astrocytes' role in regulating neurotransmitter levels had long been suspected because of the extensive wrapping of astrocytic processes around synaptic nerve endings (Peters and Palay, 1965; Bunge, 1970). As a result of numerous studies it has become apparent that astrocytes play a critical role in the uptake, inactivation and release of the amino acid neurotransmitters (Henn and Hamberger, 1971;

Neal and Iversen, 1972; Ehinger, 1972; Henn *et al*, 1974; Schrier and Thompson, 1974; Iversen and Kelly, 1975; Harber and Hutchinson, 1976; Schousboe, 1978; Hertz, 1979). Astrocytes are now thought to modulate neuronal function by controlling the synaptic levels of glutamate (GLU) and aspartate excitatory neurotransmitters as well as gamma-aminobutyric acid (GABA) and glycine inhibitory neurotransmitters (Watkins, 1973; Curtis and Johnson, 1974; Davidson, 1976; Hertz, 1979; Johnson, 1978). The biochemical involvement of astrocytes in this regulatory process consists of a glutamate-glutamine shunt. In this process the neurotransmitters GLU and GABA , which are released by presynaptic and postsynaptic nerve endings, are inactivated by active and passive cellular uptake mechanisms (Van Den Berg and Garfinkel, 1971; Van Den Berg, 1972; Benjamin and Quastel, 1975; Henn and Hamberger, 1971; Schousboe *et al*, 1977, 1978, 1981; Schousboe, 1981; Waniewski *et al*, 1986). These neurotransmitters are converted to glutamine following uptake by astrocytes (Schousboe *et al*, 1977a, 1977b). Glutamine is passively released by astrocytes for use by neurons, where it is metabolized to replenish GLU and GABA transmitter pools (Hertz and Schousboe, 1986).

Astrocytes are also believed to play a role in the metabolism of short-chain fatty acids and glycogen (Cremer *et al*, 1975; Ibrahim, 1975; Volpe and Marasa, 1977; Varon and Somjen, 1979). Astrocytic swelling is an early event which occurs following periods of ischemia (Kimelberg and Ransom, 1986). Polyunsaturated fatty acids (PUFAs) are rapidly released from membrane phospholipids during brain eschemia (Brazen, 1970). PUFAs cause numerous cellular changes including changes in the fluid domain of membranes, uncoupling of mitochondrial respiration, inhibition of Na+ and K+ATPase and the generation of oxygen free radicals and other lipid peroxides (Klausner *et al*, 1980; Hillered and Chan, 1987; Chan *et al*, 1983). Therefore, changes in

the metabolism and processing of short-chain fatty acids could have a detrimental effect on membrane integrity, protein cross-linking and DNA. In addition, these short chain fatty acids and PUFAs influence astrocyte uptake of the amino acid neurotransmitters (Chan *et al*, 1983; Yu *et al*, 1986, 1987). Glycogen levels in astrocytes are found to increase when glutamine generation is inhibited (Folbergrova *et al*, 1969) The first sign of metabolic imbalance affecting astrocytes may also be recognized by increased levels of glycogen storage.

Astrocytoma Treatment

The most essential treatment of astrocytomas and other brain tumors continues to be surgical removal. However, in recent years radiotherapy and chemotherapy have also played important roles. There are many reports indicating improved therapeutic results using combinations of radiotherapy and chemotherapy (Suzuki, 1988). Chemotherapy is composed of chemical pharmaceutics which act as either cytotoxic or cytostatic agents. Newer treatments involving immunotherapy or reverse transforming agents are presently in the experimental stage. The goal of immunotherapy is to stimulate the immune system to attack and destroy tumor cells through the use of biological response modifiers. The goal of reverse transforming agents is to control tumor cells by the action of potent differentiation promoters. NGF, consistent with its action on PC12 cells, has become the most popular reverse transforming agent for experimental use against astrocytomas.

All treatments of tumors have been directed at completely eliminating transformed cells. However, reverse transforming agents have been suggested for potential drug development based on their ability to differentiate neoplastic cells. It had logically been thought that if tumor cells

could be made to resemble differentiated cell types they would also behave normally. That is, they would cease to proliferate in an uncontrolled fashion and lose their transformation characteristics. Due to the reverse transforming effects of these agents on tumor cells, it was suspected that these agents could also cause tumor regression. However, most of the differentiation effects produced by reverse transforming agents appeared to be directed only at the cell membrane and induce a variation of immediate and delayed responses responses (Pollock *et al.*, 1990). Agents are needed that induce a prolonged response and stimulate tumor cells to differentiate towards the end-stage of the normal comparative cell's developmental sequence and become a quiescent normal functioning cell type. These expectations have made it necessary to investigate and compare the effects of these agents on the function of differentiated tumor cells as well as normal astrocytes.

Is NGF just a promoter of morphologic differentiation in astrocytes?

This dissertation investigates the effects of NGF on proliferation and function as well as morphology of astrocytoma cells. These studies are undertaken from a comparative view, rat T-9 anaplastic astrocytoma cells are compared to neonatal rat astrocytes. Proliferation is studied by characterizing cells in the cell cycle using single-stranded to double-stranded DNA and analyzing the effects of NGF by flow cytometry. Morphology is studied by light-microscopy using morphology as well as intermediate filament expression as a means of evaluating differentiation promotion following NGF treatment. Function and the effects of NGF on function are studied by determining the cellular uptake of glutamate (GLU) and γ-aminobutyric acid (GABA) before and after treatment with NGF. GLU and GABA are the major excitatory and inhibitory amino acid neurotransmitters, respectively. It has

been suggested that the increased incidence of seizures that accompany astrocytomas may be related to a disruption of normal function. Therefore, GLU and GABA uptake was determined and compared between anaplastic astrocytoma cells and neonatal astrocytes both before and after NGF treatment. The results presented here support the development of NGF as a therapeutic agent for the treatment of anaplastic astrocytomas and seizures related to this condition.

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Chapter 2

The *in vitro* Generation of an Interphase, Non-cycling, G_0 Subpopulation of Anaplastic Astrocytoma Cells Induced by Nerve Growth Factor

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Abstract

The simultaneous measurement of single stranded and double stranded DNA by using flow cytometric techniques has made it possible to distinguish subpopulations of proliferating and non-proliferating cells. The *in vitro* growth of neonatal astrocytes in a chemically defined medium (CDM) and subsequent treatment with fetal calf serum (FCS) induced the generation of a separate proliferating cell population of astrocytes. The *in vitro* growth of anaplastic astrocytoma cells in CDM and subsequent treatment with nerve growth factor (NGF) (500 *n*g/ml) induced the generation of a separate non-proliferating, quiescent G_0 subpopulation of interphase astrocytoma cells. NGF treatment failed to generate a subpopulation of neonatal astrocytes and FCS treatment failed to generate a subpopulation of astrocytoma cells. Therefore, the mechanism of induction for the generation of subpopulations of astrocytes and astrocytoma cells differ markedly. As a result, this report supports the potential development of NGF as a therapeutic agent for the treatment of astrocytomas.

Running Title: NGF Generates Non-cycling G0 Subpopulation in Astrocytoma Cells.

Key Words: Nerve growth factor, neonatal astrocytes, astrocytoma cells, flow cytometry, cell cycle analysis

Introduction

The concept that tumors are derived from a heterogeneous cell population composed of both actively cycling and non-cycling cells has been debated for years (Mendelson, 1962). However, the phenomenon of cell heterogeneity is shared between tumor cell and normal neonatal precursor cell populations (Raff *et al.*, 1983; Levi *et al.*, 1986).

Fibrous astrocytes and protoplasmic astrocytes, which differ in their morphological characteristics, but not in karyotype, have been reported to derive from a common undifferentiated astrocytic precursor cell population (Raff *et al.*, 1983; Levi *et al.*, 1986). These astrocytes derived from this precursor population are also found to differ in their *in vitro* growth rate, responsiveness to hormones and growth factors, as well as in antigen expression (Raff *et al.*, 1983). Likewise, cloned tumor cells from a single homogeneous appearing tumor have also been reported to be heterogeneous in their *in vitro* growth rate, as well as in karyotype, antigen expression, metastatic and invasive potential, immunogenic potential and responsiveness to chemotherapeutic agents (Hankansson and Trope, 1974; Pimm and Baldwin, 1977; Fidler and Kripke, 1977; Steel, 1977).

The ability to grow and reproduce is a fundamental property of most cell populations. Cells are expected to grow and proliferate in an orderly and regulated fashion. Normal cells, as well as tumor cells, grow and proliferate by cells entering a cell cycle. The cell cycle consists of two distinct periods, interphase and division, and is represented by G_1, S, G_2 and M phase components. The interphase and division periods can be distinguished as separate subpopulations of proliferating cells. The G_1 and G_2 components

represent cells in the intervals between DNA replication and mitosis. S phase is the period of DNA synthesis and M phase is the period of DNA division, which defines mitosis. There is also a G_0 component, which represents a subpopulation of non-cycling cells, whose growth was arrested in interphase. Cells which make up this non-cycling G_0 component of a normal heterogeneous population may also be considered to be a component of a quiescent pool, a separate and distinct subpopulation of the interphase cell population. Therefore, cycling cells have an interphase subpopulation which is represented by G_0, G_1 and a subpopulation during the period of division which is represented by G_2+M. Non-cycling cells are represented by a distinct quiescent subpopulation also represented by G_0, G_1 components which is derived from the interphase cells.

Unlike normal cells, which can freely enter the G_0 phase, tumor cells continue to divide and are assumed to exhibit only an extended G_1 phase. They have no true non-cycling cell component nor a quiescent subpopulation.

Subpopulations of cycling cells can be distinguished by their variation in DNA content. The G_2 and M subpopulation have twice the DNA of the G_0 and G_1 subpopulation. In addition, the G_0 and G_1 components and the G_2 and M components of a specific cell type are expected to share the same DNA content. Therefore, standard cell cycle analysis, utilizing total DNA, can not necessarily differentiate the subpopulation components G_0 and G_1 or G_2 and M.

Flow cytometry uses the principle of analyzing a population of cells by examining single cells, one at a time, following staining with a cell-specific or cell-component-specific fluorescent labeled dye. Flow cytometry has shown tremendous advantage in cell cycle analysis due to its speed, sensitivity and

precision. Because of these advantages, flow cytometry has become a very popular method for cytokinetic studies. Several different flow cytometric techniques incorporating the use of fluorescent dyes have been reported which are specific for DNA, RNA or protein (Arndt-Jovin and Jovin, 1977; Darzynkiewciz *et al.*, 1979a; Crissman *et al.*, 1990). It has also been reported that differential staining of DNA exists between mitotic and interphase cells (Darzynkiewicz *et al.*, 1977). Continuously dividing cells can also be easily characterized using flow cytometry by detecting changes in the cellular distribution of total DNA while cells pass through the cell cycle. In addition, flow cytometric scatter graph histograms can be generated using two parameters simultaneously (Darzynkiewicz *et al.*, 1979b; Darzynkiewicz *et al.*, 1980). These histograms are characteristic and unique for each specific cell type and are representative of the cell population's proliferation potential (Dethlefsen *et al.*, 1980).

Acridine orange (AO) is a fluorescent dye which has affinity for both DNA and RNA. AO intercalates into double-stranded regions of nucleic acids giving a green fluorescence near 530 *n*m and contrasts with its stacking on single-stranded nucleic acids, where it is found to give a red fluorescence near 640 *n*m. Fixed cells may therefore be analyzed as to their single-stranded (ss) and double-stranded (ds) DNA characteristics by elimination of cellular RNA (Darzynkiewicz *et al.*, 1976). This technique should also make it possible to compare cell populations based on gene expression, since ssDNA content correlates well with regions of gene expression.

Nerve growth factor (NGF) is a protein necessary for the development, maintenance and survival of sympathetic, sensory and specific cholinergic neurons (Levi-Montalcini and Angeletti, 1968; Hefti, 1986; Williams *et al.*, 1986). NGF has been widely recognized as an *in vitro* differentiation

promoter of glioma cells (Marushige *et al.*, 1987; Marushige *et al.*, 1989). In addition, NGF has been shown to retard the growth of anaplastic glioma cells *in vivo* and *in vitro* (Vinores and Koestner, 1980; Marushige *et al*, 1987).

The purpose of this study was to compare the characteristic AO flow cytometric histograms of neonatal rat astrocytes and rat astrocytoma cells and to distinguish induced changes in cell populations as well as subpopulations. If NGF induced growth retardation was due to an increase in a G_0-G_1 phase, AO flow cytometry should be capable of detecting such a change. This study, therefore, reports on a subpopulation of proliferating cells in neonatal astrocytes and a subpopulation of non-proliferating cells in anaplastic astrocytoma cells generated by the *in vitro* treatment of cell cultures with fetal calf serum or NGF, respectively.

Materials and methods

Materials

NGF (2.5S, grade II), Dispase (grade II) and RNase were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN,USA). Fetal calf serum (FCS) was purchased from Hyclone Sterile Systems Inc. (Logan, UT, USA). All culture media were prepared using stock solutions, chemicals and supplies from Life Technologies Inc. GIBCO Labs (Grand Island, NE, USA), Corning Glass Works Inc. (Corning, NY, USA) and Sigma Chemical Co. (St. Louis, MO, USA). Hl-1 supplement was purchased from Endotronics Inc. (Coon Rapids, MN, USA).

Neonatal astrocyte cultures

Four-day old Fischer rat pups obtained from Charles River Laboratories were used to establish cultures of cycling neonatal rat astrocytes (NR-1). The brains of four sibling rat pups were removed following euthanasia and placed in a petri dish containing warm DMEM high-glucose media. The meninges were removed and the brain stem and cerebellum separated. The cerebral tissues were minced into small pieces (<0.5 mm), combined and placed into a sterile centrifuge tube containing 5 ml of DMEM high-glucose media. This suspension of brain tissue was then centrifuged at 750 X g for a period of 10 min. The supernatant fluid was removed and discarded and replaced with 1.5 ml of Collagenase II-S (Sigma) solution (0.8%, w/v). The enzyme treatment procedure was followed by incubation of the cell mixture on a warm water bath (37^{o}C) shaker for 30 min, after which the brain tissue was completely dissociated to a cell suspension by intermittently pipetting with a sterile glass

pasteur pipette supplied with a cotton filter. At no time during preparation of these cultures were the cells allowed to cool below 30°C. Following the enzyme treatment the cell suspension was centrifuged at 200 X *g* for a period of 10 min and the supernatant fluid removed and discarded. An equal amount (approx. 3 ml) of DMEM high-glucose medium containing 15% FCS was gently layered on top of the cell pellet. Then the upper 2/3 of the cell pellet was resuspended in fresh medium using gentle pipetting action. A 1 ml aliquot of this cell suspension was quickly removed and used to seed primary cultures at approximately a 1:20 split ratio (0.25 ml : 5 ml medium). These stock cultures were started and maintained in 25 cm^2 tissue culture flasks (Corning) containing 5 ml of complete medium. The medium was replaced with fresh medium the following day and replaced every 3 days thereafter. Neonatal astrocytes used in these experiments were at their 3rd passage. The astrocytic character of these cells was indicated at second and third passage not only by morphology and their ability to take up γ-aminobutyric acid, but by their content of the astrocyte-specific, glial fibrillary acidic protein. Approximately 95% of these cells were GFAP positive.

Astrocytoma cells

The rat anaplastic astrocytoma cells were supplied by Dr. A. Koestner of the Department of Pathology, Michigan State University, East Lansing, MI. from his cell storage bank. The T-9 cell line originated from a high grade-anaplastic astrocytoma induced in Fischer rats by treatment with N-methyl-N-nitrosourea (MNU) (Schmidek *et al.*, 1971). Stock cultures of T-9 were established in 25 cm^2 tissue culture flasks and maintained in complete serum-supplemented medium (DMEM and 10% FCS) with replacement every 3rd day and the splitting of cells every 6th day at a 1:100 split ratio.

Culture conditions

Cells were cultured in a Hotpack CO_2 Incubator which was maintained at 5% CO_2, 37°C and constant humidity. Both NR-1 and T-9 cells were split into 25 cm^2 tissue culture flasks containing 5 ml of complete medium. The complete medium was changed the following day and replaced with either of two chemically defined media (CDM). CDM HL1A was composed of DMEM supplemented with 1% (v/v) HL-1 supplement, 400μM glutamine, gentamycin at 10 μg/ml, glucose at 1 mg/ml, $CaCl_2$ at 175 μg/ml and $MgSO_4$ at 125 μg/ ml. CDM HL1B was also composed of DMEM and contained the same supplementation as HL1A but also contained hydrocortisone at 1.6 μg/ml, prostaglandin F2-alpha at 440 *n*g/ml, putrescine at 78 μg/ml, basic-fibroblastic growth factor at 8.8 *n*g/ml and myelin basic protein at 440 *n*g/ml. This HL1B medium was a slight modification of that originally proposed by Morrison and De Vellis in 1981 as a CDM that initiates differentiation of astrocytes. HL-1 supplement contained 29 μg/ml total protein with 15 μg/ml insulin and contained no additional growth factors.

Cell culture treatment

NR-1 and T-9 cells used in these studies were seeded at 6-12,000 cells/ml into 5 ml of complete, serum-supplemented medium at initial plating. The medium was changed the following day to serum-free CDM. Three days later the CDM was replaced with fresh CDM of the same type and NGF or serum added. NGF was added at 500 *n*g/ml or serum added at 10% (500 μl/5 ml medium). All cell cultures were treated with NGF or serum for 48 hrs and colcemide (0.1 μg/ml) for 3 hrs prior to cell fixation.

Cell harvesting and fixation

Following cell culture treatment, the medium was removed and cells were incubated in 2 ml of Dispase (2.4 U/ml) for a period of <30 sec. Dispase II was removed and cells were incubated in 2 ml of trypsin (0.05% trypsin, 0.53mM EDTA) for a period of <30 sec. Trypsin was removed and cells were collected in 4 ml PBS (140mM NaCl, 12mM Na_2HPO_4, 3.5mM NaH_2PO_4, pH 7.4). The cells were freed from the flask by gentle pipetting action and worked into a single cell suspension. The contents of two identically treated flasks were pooled into 15 ml sterile centrifuge tubes and the cell suspension was centrifuged for 10 min at 750 X g. The supernatant fluid was removed and discarded. Cells were resuspended into a single cell suspension following the addition of 1 ml of Hank's balanced salt solution (HBSS) without phenol red. This cell suspension was again centrifuged for 10 min at 750 X g. The supernatant fluid was dicarded and cells resuspended in 1 ml HBSS as before. This single cell suspension was then rapidly fixed in a sterile glass centrifuge tube by placing cells into ice cold acetone-alcohol fixer (100% acetone, 80% ethanol; 1:1, v/v). Cells were stored in the cold following fixation. Preparation and staining of cells was carried out just prior to flow cytometry.

Cell Preparation and staining for flow cytometry

Cells suspended in ice-cold fixative were centrifuged for 10 min at 750 X g. The fixer was removed and discarded. The cell pellet was resuspended in 1 ml of HBSS without phenol red. This cell suspension was centrifuged and resuspended in fresh HBSS. The cell suspension was then treated with RNase (500 Kunitz units) and incubated in a water bath shaker for 60 min at 37°C. The incubation was stopped by centrifuging at 750 X g for 10 min and pouring off the RNase containing supernatant fluid. The cells were

resuspended and rinsed one more time in 1 ml of HBSS and then centrifuged and resuspended in HBSS, as before. An aliquot of 0.2 ml of cell suspension (5-10 X 10^5 NR-1 cells and 1-3 X 10^6 T-9 cells) was then mixed with 0.5 ml of 0.1M KCl-HCl buffer (0.2M KCl, 0.2M HCl; 1:1, v/v, pH 1.4) for 30 sec just prior to the addition of 2 ml AO (6 μg/ml) solution (0.2M Na_2HPO_4, 0.1M citric acid, pH2.6) at room temperature (20-25^{o}C). Similar techniques have been described elsewhere (Darzynkiewicz *et al.*, 1976; Trangos *et al.*, 1977; Darzynkiewicz *et al.*, 1986). Flow cytometric analyses were run as soon as possible after cell preparation and staining.

Flow cytometric fluorescence measurements

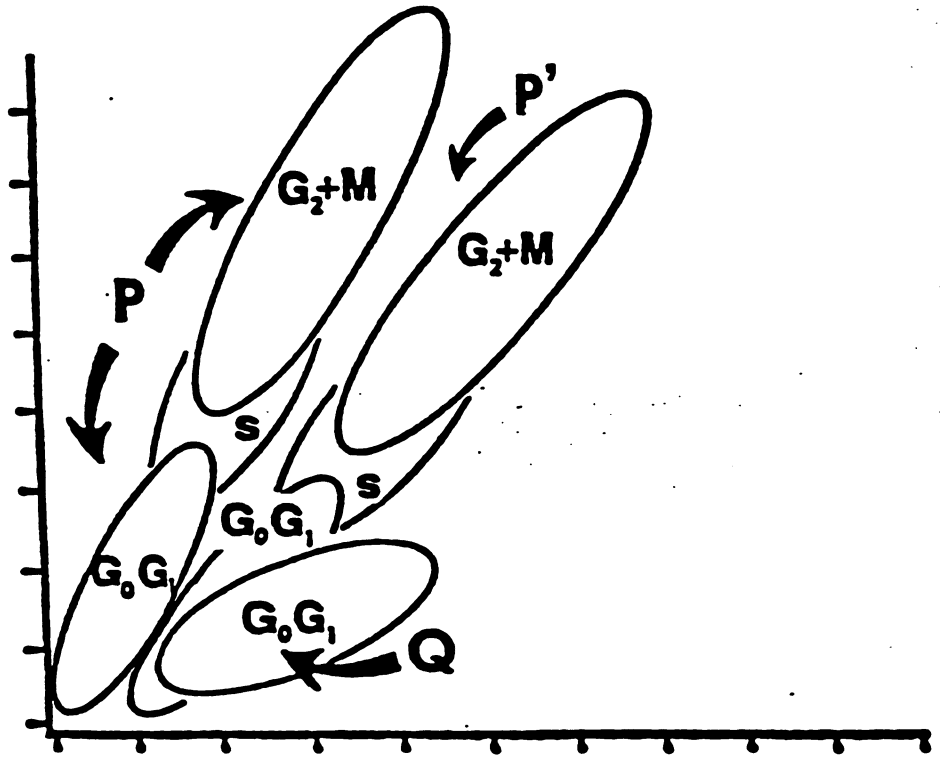
The fluorescence of individual cells was measured in a 50-H cytofluorograph (Ortho Diagnostics-BD). A blue light (488 *n*m) source was used to excite red fluorescence at 640 *n*m and green fluorescence at 530 *n*m. Scatter graph histograms were generated and recorded. All studies were done on a minimum of three separate cultures and were qualitatively but not quanitatively analyzed to determine proliferative and non-proliferative populations and subpopulations. The coefficient of variation in these cell population studies was consistently <1%.

Results

The scatter graph histograms of **NR-1 neonatal astrocytes** were characteristic of a single, uniform, proliferative cell population following culture in complete serum-supplemented medium or when cultured in CDM (Figure 1, 2A, 3A). Cells cultured in CDM and then treated with NGF (500 *n*g/ml) did not markedly differ in their histogram from those cultured without treatment (Figure 3B). However, NR-1 cells cultured in CDM and then treated with serum (100 μl/ml) were found to generate two parallel populations of cycling cells (Figure 3C).

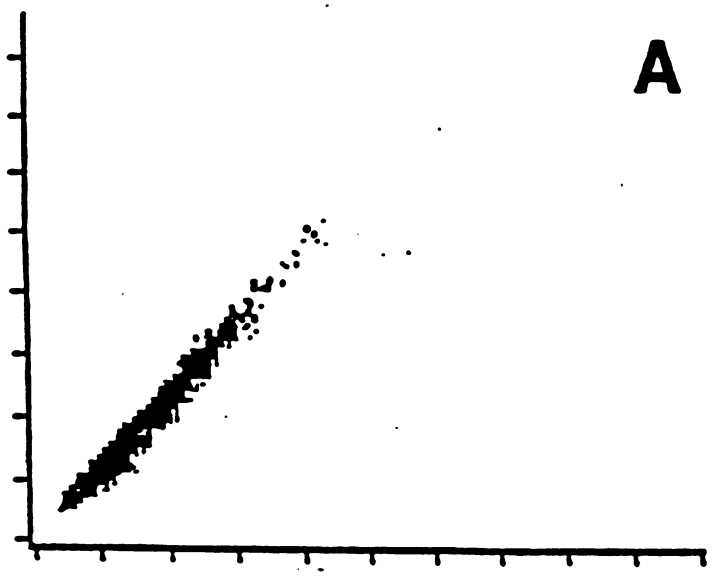
The scatter graph histograms of **T-9 anaplastic astrocytoma cells** were characteristic of a uniform, proliferative tumor cell population when cultured in complete serum supplemented medium or when cultured in CDM (Figure 1, 2B, 4A). Cells cultured in CDM and then treated with serum (100 μl/ml) gave the same scatter graph histogram characteristic of a cycling-cell population (Figure 4C). However, T-9 cells cultured in CDM and then treated with NGF (500 *n*g/ml) showed two subpopulations of cells, a cycling and non-cycling population (Figure 4B).

Figure 1 Schematic illustration of a fluorochromatic scatter graph histogram of proliferative and quiescent pools of cycling and non-cycling cell populations, following fixation and AO staining. The Y axis represents dsDNA and the X axis ssDNA. Cycling cell populations have scatter graph histograms characterized by G_0-G_1, S and G_2+M components of the cell cycle. This is illustrated by the sequence of subpopulations making up the proliferative pools P and P', on the far left. Interphase cells of the quiescent pool are non-cycling and therefore, do not have S or G_2+M components. They are illustrated by the Q subpopulation on the right, which contains only non-cycling G_0 and G_1 components.



RED-FL(X) VS GRN-FL(Y)

Figure 2. Scatter graph histograms of AO stained cycling cell populations. ***NR-1 neonatal astrocytes*** **(2A)** and ***T-9 anaplastic astrocytoma cells*** **(2B)** were cultured in complete FCS-supplemented (10%) medium and illustrated proliferative populations of cells..


A
RED-FL(X) VS GRN-FL(Y)

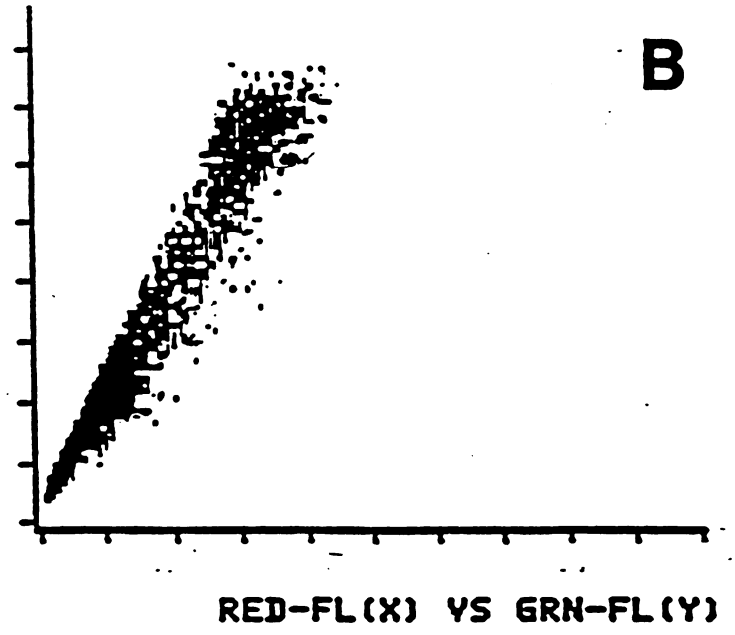
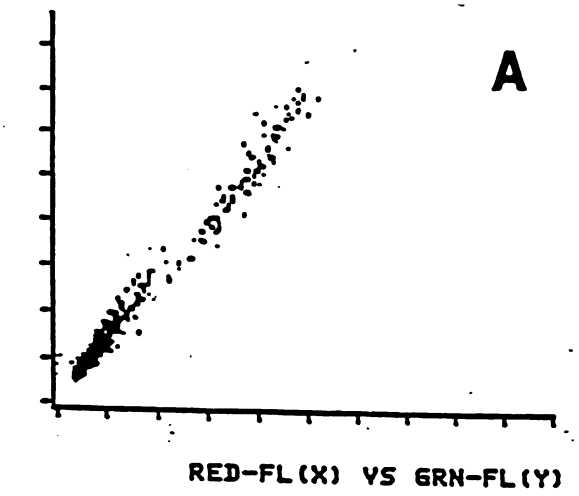
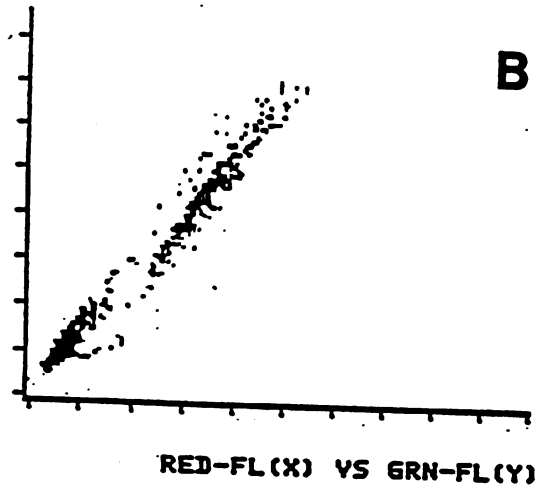

B
RED-FL(X) VS GRN-FL(Y)

Figure 3. Scatter graph histograms of AO stained NR-1 astrocytes. NR-1 cells were cultured in HL1A CDM with ***no treatment*** **(3A)**, following treatment with ***NGF (500 ng/ml)*** **(3B)** or following treatment with ***FCS (100 µl/ml)*** **(3C)**. Two parallel proliferative cell populations are illustrated in figure 3C.


A
RED-FL(X) VS GRN-FL(Y)


B
RED-FL(X) VS GRN-FL(Y)

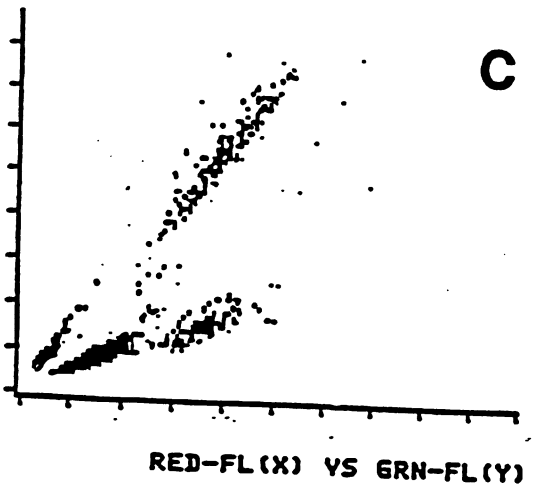
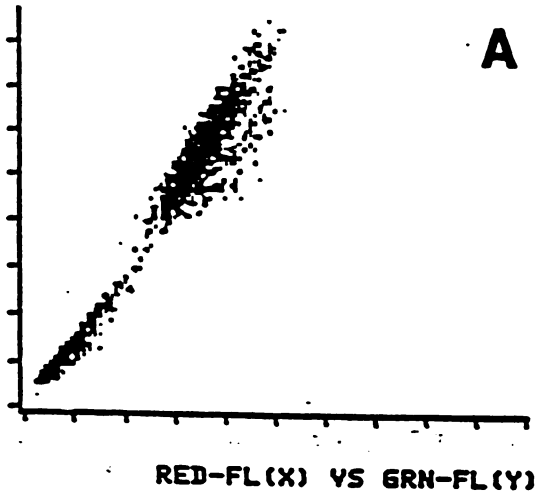
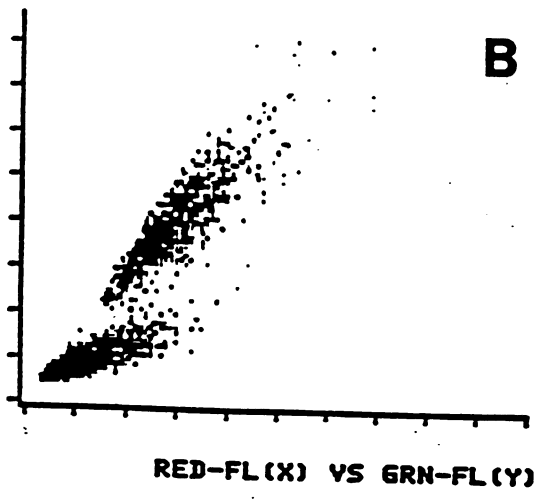
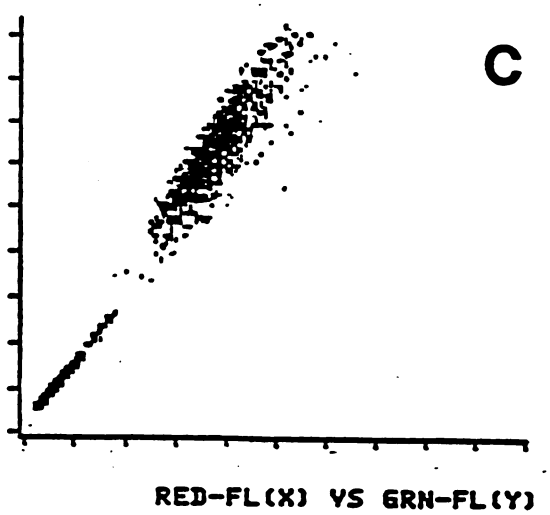

C
RED-FL(X) VS GRN-FL(Y)

Figure 4. Scatter graph histogram of AO stained T-9 astrocytoma cells T-9 cells were cultured in HL1B CDM with ***no treatment*** **(4A),** following treatment with ***NGF (500 ng/ml)*** **(4B)** or following treatment with ***FCS (100 μl/ml)*** **(4C)**. The generation of a non-proliferative G_0 interphase cell population, induced by NGF treatment, is illustrated by formation of a quiescent pool of cells in figure 4B.


A
RED-FL(X) VS GRN-FL(Y)


B
RED-FL(X) VS GRN-FL(Y)


C
RED-FL(X) VS GRN-FL(Y)

Discussion

Neoplastic transformation is well-recognized as a process involving initiation and promotion which generates an aberrant proliferating cell population. The results of this two step-process has been termed carcinogenesis. Carcinogenesis can be further defined as a cellular generated escape from growth and organizational control. These cellular changes are recognized by pathologists as changes in phenotype of individual cells and tissues. The initiation of this process is accepted to be due to genetic lesions brought about by oncogenic viruses, chemical carcinogens, inherited mutant genes as well as spontaneous mutations acquired both pre- and post-natally (Weinstein, 1988). Neoplastic cells generated in this process simply proliferate, unrestrained by normal physiologic control mechanisms. Cells continue to grow unrestrained and invade adjacent tissues. Normal cell communication, necessary for cellular organization and organogenesis, appears to be interupted (Pitts *et al.*, 1987; Yamasaki, 1990). As a result of carcinogenesis, organ systems malfunction, aberrant cells invade and colonize in inappropriate locations and disease processes ensue (Patterson, 1974; Weinstein, 1988).

The majority of strategies developed for cancer therapy have involved cytotoxic agents that kill neoplastic cells by interacting directly with their DNA. Other agents have been developed that are cytostatic and stop cells in a specific phase of the cell cycle. The problems involved with either of these therapeutic regimens are their effects on normal cell populations. Cytotoxic drugs developed to kill proliferative tumor cell populations also kill normal

proliferative cell populations. Cytostatic drugs that stop proliferative cell populations in a specific phase of the cell cycle also affect the DNA of normal cells and are responsible many times for the initiation of drug-induced carcinogenesis. However, the main priority in the development of any efficacious anti-neoplastic agent is still to control proliferation.

The measurement of DNA using flow cytometric techniques has made it possible to distinguish subpopulations of cells in various phases of the cell cycle (Darzynkiewicz *et al.*, 1979; Dethlesen *et al.*, 1980; Nusse *et al.*, 1990). In addition, cycling cells of proliferative populations can be differentiated from non-cycling cells of non-proliferative cell populations (Dethlefsen *et al.*, 1980). However, problems have existed in differentiating closely related cell populations. Normal cells of the same karyotype and tumor cells expressing aneuploidy have made it difficult to distinguish pools of proliferative and non-proliferative populations as well as their subpopulation components. However, differences in the sensitivity of DNA to acid denaturation and staining with AO have made it possible to differentiate subpopulations of cycling cells, as well as non-cycling cells (Darzynkiewicz *et al.*, 1979b). Separate differentiated cell types, though even closely related, are known to express different genes. Gene expression is related to ssDNA regions of the genome. Therefore, different cell types can be expected to vary in ssDNA content and location. As a result, flow cytometry may use the dual flurochromatic properties of AO staining to differentiate ssDNA from dsDNA, if RNA is eliminated. As a result, different cell populations can be quickly and easily characterized by their ss- and dsDNA content and distinguished from one another (Figure 1).

Neonatal astrocytes have been reported to have the capacity to differentiate morphologically into two different end-stage types of astrocytes

(Raff *et al.*, 1983; Levi *et al*, 1986). Therefore, neonatal astrocytes have been regarded as a precursor cell population which has the capacity to proliferate *in vitro* and to respond to differentiation factors. Astrocytes have been found to differentiate morphologically to either stellate cells with a low proliferation capacity or to epithelioid cells which continue to proliferate similar to the precursor cells (Raff *et al.*, 1983). These two differentiated astrocytes have the characteristics of fibrous and protoplasmic astrocytes, respectively. Since the cells isolated for use in this study were astrocyte precursor cells we can expect them to differentiate to either protoplasmic or fibrous astrocytes (Bailey and Cushing, 1926). This study has characterized these astrocytic cell types based on their ss and dsDNA. Therefore, we speculate that protoplasmic astrocytes, with a greater proliferation potential, are illustrated by the scatter graph histogram in Figure 3C as the cell population on the left, while the fibrous astrocytes are illustrated as the population on the right. As a result, AO flow cytometry has the ability to differentiate two closely related cell populations of the same karyotype, but which differ slightly in gene expression and proliferation potential.

NGF has been reported to retard the growth rate of *in vivo* and *in vitro* astrocytoma cells (Vinores and Koestner, 1980; Marushige *et al.*, 1987). Although the physiologic consequences of the NGF-cell interaction is known with regards to morphologic differentiation of sympathetic neurons, very little is known about the molecular mechanisms responsible for the retardation of growth of astrocytoma cells. In this study NGF was not found to markedly affect the proliferating pool of neonatal astrocytes (Figure 3B). However, NGF was found to induce a quiescent pool of astrocytoma cells (Figure 4B). Red metachromatic fluorescence of AO-ssDNA complexes has been reported to be highest in quiescent cells, while the orthochromatic green

fluorescence of dsDNA complexes has been found to be greatest in cycling cell populations (Darzynkiewicz *et al.*, 1978). This suggests that NGF may preferentially act to increase the sensitivity of chromatin DNA in astrocytoma cells to acid-denaturation. Therefore, AO flow cytometry has the potential to differentiate a proliferative from a non-proliferative tumor cell population.

The significance of this study is that NGF reduces the growth potential of astrocytoma cells while not affecting neonatal astrocyte populations *in vitro*. These results therefore support the potential use of NGF as a therapeutic agent for the treatment of anaplastic astrocytomas.

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Chapter 3

Induction of Morphologic Differentiation in Astrocytes and Astrocytoma Cells by Nerve Growth Factor.

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Abstract

An *in vitro* effect of nerve growth factor (NGF) on end-stage morphologic differentiation of neonatal astrocytes and anaplastic astrocytoma cells of the rat is reported. NGF (100 *n*g/ml) treatment of cell cultures in the presence of 25μM glutamate (GLU) results in morphologic differentiation of cells resembling protoplasmic and fibrous astrocytes. These results are markedly different than previous reports describing cells treated with NGF (5 μg/ml) under much different conditions. Treatment of astrocytoma cells with either NGF (500 *n*g/ml) or 25μM GLU alone did not induce morphologic differentiation. This report concerns the potential use of NGF as a pharmacologic agent for the differentiation and arrest of tumor growth.

Running Title: The effect of NGF on astrocyte morphology

Key Words: nerve growth factor, glutamate, astrocytes, astrocytoma cells chemically defined medium.

Introduction

Astrocytes are classified on the basis of their morphology as either epithelioid protoplasmic (Type 1) or stellate fibrous (Type 2) astrocytes. Most isolates of neonatal astrocytes from brain tissue have been found to contain a mixture of these two cell types. Most anaplastic astrocytoma cells lose all morphologic identity with astrocytes and in culture rapidly take on a monotonous spindle-like shape forming a confluent sheet of cells.

Nerve growth factor (NGF) is a widely recognized potent differentiation promoter of sympathetic and sensory neuronal cell populations as well as pheochromocytoma and neuroblastoma cells (Greene and Tischler, 1976; Reynolds and Perez-Polo, 1981; Sonnefield and Ishii, 1982). In addition, NGF has been characterized as an important factor in the maintenance and survival of neuronal cell populations (Whittemore and Seiger, 1987; Thonen *et al.*, 1987; Springer, 1988). NGF and other maturation factors have also been found to induce morphologic differentiation in glioblasts and glioma cells (Lim, 1980; Marushige *et al.*, 1987, 1989). Although the molecular mechanisms by which NGF promotes neuronal differentiation are unknown, previous studies have suggested that phosphorylation of specific proteins occurs during the differentiation process (Hashimoto, 1988; Koizumi *et al.*, 1988; Hashimoto and Hagino, 1989). The cytoskeleton is considered to be the major factor responsible for cellular expression of shape. Variations in cytoskeletal components, therefore, would give rise to variations in shape, morphologic characteristics and function, since morphology and function of specific cell populations tend to go hand in hand.

Studies in the past have attempted to reverse the anaplastic character of neoplastically transformed astrocytes by treatment with various differentiation promoters (Marushige *et al.*, 1987). These studies were designed to regain normal end-stage cell differentiation. To obtain this goal treatment with large doses of promoters was used over a period of 2-4 days.

This study reports on the immediate effects of NGF, at a lower dose, to induce morphologic differentiation in anaplastic astrocytoma cells cultured in a chemically defined medium(CDM) in the presence of the excitatory neurotransmitter glutamate (GLU).

Methods

Materials

NGF (2.5S, grade II) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN,USA). Fetal calf serum (FCS) was purchased from Hyclone Sterile Systems Inc. (Logan, UT, USA). All culture media were prepared using stock solutions, chemicals and supplies from Life Technologies, Inc,GIBCO Labs (Grand Island, NE, USA), Corning Glass Works Inc. (Corning, NY, USA) and Sigma Chemical Co. (St. Louis, MO, USA). Hl-1 supplement was purchased from Endotronics Inc. (Coon Rapids, MN, USA) and rats from Charles Rivers Labs. (Portage, MI, USA). L-Glutamate (GLU) was purchased from ICN-Biomedicals Inc. (Costa Mesa, CA, USA).

Neonatal Rat Astrocytes

Neonatal rat astrocytes were generated using 4-day-old Fischer rat pups. The brains of four rat pups were removed following euthanasia and placed in a petri dish containing warm DMEM high glucose media. The meninges were removed and the brain stem and cerebellum were separated from the cerebrum. The cerebral tissues were minced into small pieces (<0.5 mm), placed into a sterile centrifuge tube containing 5 ml of DMEM high-glucose media. This suspension of brain tissue was then centrifuged at 750 X g for a period of 10 min. The supernatant fluid was removed and discarded and replaced with 1.5 ml of Collagenase II-S (Sigma) solution (0.8%, w/v). The enzyme treatment procedure was followed by incubation of the cell mixture on a warm water bath (37^{o}C) shaker for 30 min, after which the brain tissue

was completely dissociated into a cell suspension by intermittently pipetting with a sterile glass pasteur pipette supplied with a cotton filter. At no time during preparation of these cultures were the cells allowed to cool below 30°C. Following the enzyme treatment the cell suspension was centrifuged at 200 X *g* for a period of 10 min and the supernatant fluid removed and discarded. An equal amount (approx. 3 ml) of DMEM high-glucose medium containing 15% FCS was gently layered on top of the cell pellet. Then the upper 2/3 of the cell pellet was resuspended in fresh medium using gentle pipetting action. A 1 ml aliquot of this cell suspension was quickly removed and used to seed primary cultures at an approximately a 1:20 split ratio (0.25 ml: 5 ml medium). These stock cultures were started and maintained in 25 cm^2 tissue culture flasks (Corning) containing 5 ml of complete medium. The medium was replaced with fresh nutrient medium the following day and replaced every 3 days thereafter. Neonatal astrocytes used in these experiments were at their 3rd passage. The astrocytic character of these cells was indicated at second and third passages not only by morphology, and their ability to take up γ-aminobutyric acid, but by their content of the astrocyte-specific cell marker, glial fibrillary acidic protein (GFAP).

Astrocytoma Cells

The rat anaplastic astrocytoma cells were supplied by Dr. A. Koestner of the Department of Pathology, Michigan State University, East Lansing, MI. from his cell storage bank. The T-9 cell line originated from a high grade-anaplastic astrocytoma induced in Fischer rats by treatment with N-methyl-N-nitrosourea (MNU) [31]. Stock cultures were established in 25 cm^2 tissue culture flasks and maintained in complete serum supplemented medium

(DMEM and RPMI 1640/Hams F12) replacement every 3rd day and the passage of cells every 6th day at a 1:100 split ratio (1-2 X 10^3 cells).

Culture Conditions

Cells were cultured in a Hotpack CO_2 Incubator which was maintained at 5% CO_2, 37°C and constant humidity. Both neonatal astrocytes and astrocytoma cells were split into multi-well (6 well) culture plates (GIBCO Labs) containing 3 ml of complete medium. This medium helped to assure cell attachment. The complete medium, serum supplemented, was changed the following day and replaced with chemically defined medium (CDM). The CDM was composed of DMEM supplemented with 1% (v/v) HL-1 supplement, 400μM glutamine, gentamycin at 10 μg/ml, glucose at 1 mg/ml, $CaCl_2$ at 175 μg/ml and $MgSO_4$ at 125 μg/ ml. and in addition contained hydrocortisone at 1.6 μg/ml, prostaglandin F2-alpha at 440 *n*g/ml, putrescine at 78 μg/ml, basic-fibroblastic growth factor at 8.8 *n*g/ml and myelin basic protein at 440 *n*g/ml. This CDM medium was a slight modification of that originally proposed by Morrison and De Vellis in 1981 as a CDM that initiates differentiation of astrocytes. HL-1 supplement contains 29 μg/ml total protein with 15 μg/ml insulin and contains no additional growth factors or glutamate.

Morphologic Effects Study of NGF and GLU

Neonatal astrocytes and astrocytoma cells used in this study were seeded at 6-12,000 cells/ml into 3 ml of complete, serum-supplemented, medium at initial plating. The medium was changed the following day to serum-free CDM. Three days later the CDM was replaced with fresh CDM and NGF (500 *n*g/ml) or glutamate (25μM) was added to test the single effects of

NGF and GLU. Cells were incubated for 48 hr following treatment and then photomicrographed. Three or more separate cultures were used as replicates for each study.

To test the immediate combined effects of NGF and GLU on astrocytes and astrocytoma cells the preceding protocol was followed with the exception that there were no treatments until the fifth day of culture in CDM. The cells were treated with 25μM GLU and incubated for 10 min at room temperature (20-25^{o}C). The cells were treated with NGF (100 ng/ml) for 3 min near the end of the GLU incubation (from the 7 min point of the 10 min GLU incubation). The medium was then removed and the cells were rinsed in a HEPES buffered saline pH 7.4, also containing 25μM GLU for 10 min. The buffer was removed and the cells were acid-fixed with 1M perchloric acid for 10 min. The fixer was removed and the cells were covered with Tris buffer and photographed in the culture dishes using an inverted Nikon TMS microscope equipped with a 35 mm Nikon FGW camera.

Results

The immediate, combined effects of NGF (100 *n*g/ml for 3 min) and GLU (25μM for 10 min) on morphologic differentiation in cultures of undifferentiated neonatal rat astrocytes and rat anaplastic astrocytoma cells are illustrated in Figures 1A and 1B, respectively. This combined treatment resulted in these cells differentiating towards end-stage astrocytes exhibiting morphologic characteristics of protoplasmic and fibrous astrocytes. The morphologic characteristics of astrocytoma cells and neonatal rat astrocytes cultured in CDM are illustrated in Figure 2A and 3A, respectively. In addition the effects of treatment on these individual cell types with GLU (25μM for 48 hrs) or NGF (500 *n*g/ml for 48 hrs) alone cultured in CDM are illustrated in Figures 2B, 2C and 3B, 3C, respectively. Neither NGF nor GLU treatment alone markedly affected morphologic differentiation by astrocytoma cells. However, there was a difference in the response of neonatal astrocytes to GLU and NGF. In response to NGF neonatal astrocytes took on greater epithelioid differentiation, this was in contrast to their response to GLU, where cells appeared to contract and exhibit a condensed cytoplasm and nucleus. The plasma membrane and golgi apparatus of the astrocytoma cells were also quite noticeable in the acid-fixed cell preparations (Figure 1B).

Figure 1. Morphologic differentiation of astrocytes and astrocytoma cells by NGF *in vitro*. Morphologic differentiation of ***neonatal rat astrocytes*** **(1A)** cultured in CDM and treated with NGF/GLU (100 *n*g/ml & 25μM). *Solid arrowhead* identifies protoplasmic type astrocyte and *open arrowhead* identifies fibrous type astrocyte. Morphologic differentiation of ***rat anaplastic astrocytoma cells*** **(1B)** cultured in CDM and treated with NGF/GLU (100 *n*g/ml & 25μM). *Solid arrowhead* identifies protoplasmic type astrocyte and *open arrowhead* identifies fibrous type astrocyte. *Arrows* identify the carbohydrates, confirmed by PAS staining, of the plasma membrane and golgi apparatus. Bar in lower right represents 50 μm.

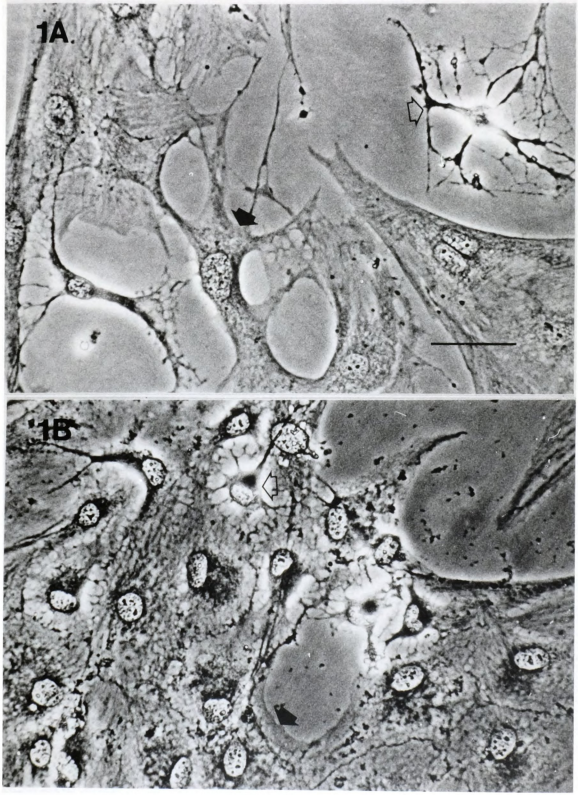

1A.
1B

Figure 2. Morphology of rat anaplastic astrocytoma cells cultured in CDM. Morphology of rat anaplastic astrocytoma cells cultured in CDM with ***no additional treatments*** **(2A),** ***treated with GLU (25μM).*** **(2B)** or ***treated with NGF (500*** **ng/ml) (2C).**

Bar in lower right represents 50 μm.

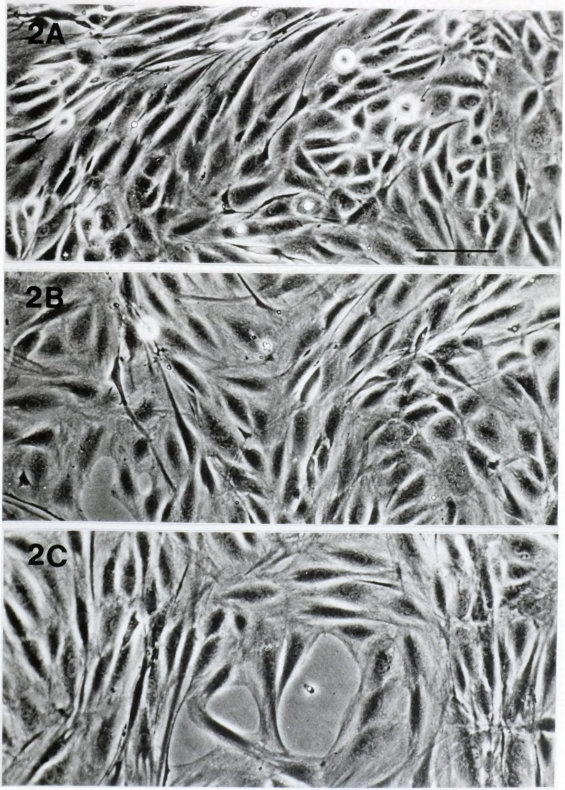
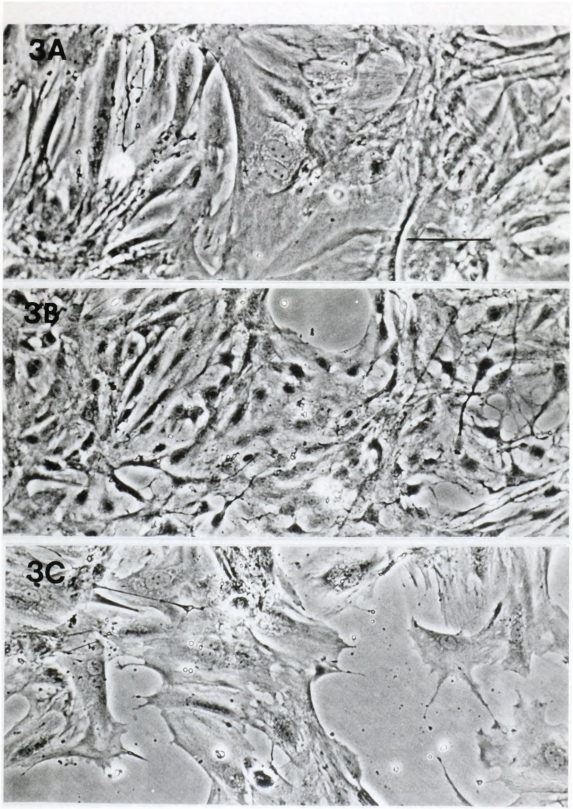

2A
2B
2C

Figure 3. Morphology of neonatal rat astrocytes cultured in CDM.
Morphology of neonatal rat astrocytes cultured in CDM with ***no additional treatments*** **(3A),** ***treated with GLU (25μM).*** **(3B)** or ***treated with NGF (500*** **ng/ml) (3C).**
Bar in lower right represents 50 μm.


3A
3B
3C

Discussion

The results presented in this report demonstrate the importance of early epigenetic events in the determination of morphologic differentiation by astrocytes. Past reports on morphologic differentiation by these types of cells have focused on cell density and changes associated with the cellular content of cytoskeletal proteins (Goldman and Chiu, 1982; Goldman and Chiu, 1984; Marushige *et al.*, 1989). Regulation of cell morphology and differentiation have likewise been reported to be associated particularly with the effects of cyclic AMP. There are many studies describing changes in cell shape following treatment with dibutyryl cyclic AMP. Many of these studies have suggested that cyclic AMP is a mediator for the morphologic effects produced by NGF (Schubert *et al.*, 1978; Garrels and Schubert, 1979; Halegoua and Patrick, 1980; Cremins *et al.*, 1986). However, most of the alterations in morphology that have been described in previous studies appear to be most pronounced in low density cultures where cells are seen to change from a flat polygonal shape to cells exhibiting a rounded shape with condensed cytoplasm and many fine cytoplasmic processes (Moonen *et al.*, 1975; Trimmer *et al.*, 1982). Cells in low density cultures have also been shown to express a high content of cytoskeletal proteins (Goldman and Chiu, 1984). This increased production of cytoskeletal proteins by cells in low density cultures is therefore suggested to be the cause for the change in morphology and differentiation seen in these cells.

Astrocytes represent a heterogeneous cell population of the brain. They are heterogeneous with respect to morphology as well as

membrane surface properties. Astrocytes are distributed in the brain relative to their specific function. There are two basic morphologic types of astrocytes, one is an epithelioid protoplasmic astrocyte (Type 1) while the other is a stellate-appearing fibrous astrocyte (Type 2) (Raff *et al.*, 1983).

Primary astrocytes *in vitro* have also been characterized as being a heterogeneous cell population (Steig *et al.*, 1980; Trimmer *et al.*, 1982). Questions have always been asked as to whether this heterogeneity resulted from an intrinsic heterogeneity of astrocytes during initial isolation or was simply the result of *in vitro* conditions. Our results suggest that astrocytes and astrocytoma cells *in vitro* both exhibit a mixture of two cell types with morphologic characteristics of protoplasmic and fibrous astrocytes. These cell types are assumed to be accurate based on previous reports in the literature on precursor cell populations and developmental sequence (Bailey and Cushing, 1926; Raff *et al.*, 1983). However, the proportion of one morphologic cell type to the other may be influenced by cell density. In addition, protoplasmic astrocytes have been reported to respond to growth factors and cyclic AMP while fibrous astrocytes have not (Herschman, 1986).

NGF is a potent differentiation promoter of pheochromocytoma cells (Greene and Tischler, 1976). NGF's effect on differentiation by these cells has been termed early and late occurring events (Greene and Tischler, 1982; Greene, 1984). Early events are characterized by their rapid onset and are many times referred to as immediate cell responses which are transcriptionally independent. Early events appear to peak around 15 min following treatment. In contrast to early events, late events are observed between 24 and 48 hr and are frequently found to be transcriptionally dependent. Early events include the generation of cyclic AMP, phosphorylation reactions and phosphoinositide hydrolysis. Late events

include neurite outgrowth by pheochromocytoma cells as well as their development of electrical excitability and the induction of several neuron specific proteins (Basi *et al.*, 1987; Karns *et al.*, 1987; Pollock *et al.*, 1990). Early events appear to be epigenetic events shared by many different cell types. Late events appear to be cell specific events regulated by gene expression. NGF has been found to induce membrane sodium channels and cyclic AMP has been found to decrease sodium current. This suggests that NGF may not act through cyclic AMP as a second messenger. Other activators of cyclic AMP cascades have also been found to inhibit NGF-induced responses (Greene *et al.*, 1986; Doherty *et al.*, 1987).

Glutamate (GLU) is an excitatory amino acid neurotransmitter that is ubiquitous to the central nervous system. Astrocytes function to take up synaptic GLU following neuronal release. GLU is also a very important constituent of brain metabolism and plays a significant role in regulating levels of ammonia in the brain. Astrocytes share two ionotrophic GLU receptors with neurons (Hosli *et al.*, 1979), and in addition express a GLU receptor associated with the hydrolysis of membrane phosphoinositides (Nicoletti *et al.*, 1986_a; Nicoletti *et al.*, 1986_b; Nicoletti *et al.*, 1987). This receptor functions in membrane signal transduction. Activation of this receptor generates 1,4,5-inositol triphosphate (IP3) and calcium (Ca^{++}) as second messengers.

Anaplastic astrocytoma cells cultured in CDM do not differentiate to end-stage astrocytes (Figure 2-A). The treatment of these cells with GLU (25μM) or NGF (500 *n*g/ml) for 48 hrs also causes no significant change in cell morphology (Figure 2B, 2C). However, the combined treatment of anaplastic astrocytoma cells with GLU (25μM) for 10 min and NGF (100 *n*g/ml) for three min induces end-stage morphologic differentiation (Figure 1B). This

response is markedly different than earlier reports from our department where cells were treated with NGF (5 μg/ml) under much different conditions (Marushige *et al.*, 1987, 1989). The morphologic response in this study illustrates individual cell variation in differentiation resembeling both protoplasmic and fibrous astrocytes. Whereas previous studies describe a uniform population change characterized by a flattened cytoplasmic projection, lamellipodia, with long slender filamentous processes, filopodia (Marushige *et al.*, 1987). The treatment of neonatal astrocytes in the same way also induces their morphologic differentiation (Figure 1A). These results suggest that NGF may play a permissive role in astrocytes which facilitates the regulation of differentiation and allows astrocytes and astrocytoma cells to express end-stage morphologic differentiation in response to other epigenetic events. In this experimental model these events are represented by the membrane signal transduction initiated by GLU. Generation of IP3 and Ca^{++} through activation of the GLU receptor could in turn lead to activation of other protein kinases and the phosphorylation of specific proteins which may in turn control the organization of the cytoskeletal system. In addition, NGF may also act to sustain this morphologic differentiation. That is, morphologic differentiation may not only not take place without NGF but cells may revert back to their pre-undifferentiated morphology with out NGF's continued presence. However, the earlier reports from our department indicated that prolonged treatment with NGF at higher doses, over 4 days, under different conditions induced the characteristic change in cell morphology previously described and that this morphologic change appeared to persist even in the absence of NGF (Marushige *et al.*, 1987).

In contrast to protein phosphorylation, which directly influences protein function and organization, glycosylation often determines specificity

of functional proteins and regulates their turnover and organizational distribution within the cell. In the past much less attention has been paid to the functional significance of glycosylation than to the effects of phosphorylation. One basic type of change in the expression of membrane carbohydrates and glycosylation, which takes place following neoplastic transformation, has been shown to be affected by NGF (McGuire and Greene, 1978). In many tumor cell populations high molecular weight fucose-containing glycoproteins appear to increase (Warren *et al.*, 1973). The treatment of pheochromocytoma cells with NGF has been found to increase the incorporation of fucose and glucosamines (McGuire and Greene, 1978). These reports suggests that NGF may have an effect on glycosylation and differentiation which is quite different from its effect to facilitate morphologic differentiation through phosphorylation. NGF may stimulate imperfect glycosylation of membrane proteins through a cellular mechanism which is non-specific, since ganglioside composition of neurons and astrocytes are different (Geissler *et al.*, 1977). This further suggests that morphologic differentiation may be modulated through two completely different mechanisms in which NGF could impart both a positive and negative effect.

The acid fixation used to prepare the NGF/GLU combination treatment of astrocytes and anaplastic astrocytoma cells identifies what may be the large complex carbohydrates contained in the plasma membrane and the golgi apparatus of the astrocytoma cells (Figure 1B). The carbohydrates were also confirmed in the astrocytoma cells by using a PAS stain. However, these carbohydrates could not be identified by acid fixation in the normal neonatal astrocytes (Figure 1A).

The significance of this study is that NGF/GLU combination treatment of astrocytes or astrocytoma cells induces morphologic differentiation towards

end-stage protoplasmic and fibrous astrocytes (Figure 1A, 1B). This response is suggested to be facilitated by NGF and may be mediated by IP3 and Ca^{++} mobilization through activation of GLU-receptor linked hydrolysis of phosphoinositide. This generation of IP3 and mobilization of Ca^{++} further leads to activation of other protein kinase systems leading to the phosphorylation of specific proteins controlling cytoskeletal organization. In addition, it is suggested that NGF may not correct the defect which leads to aberrant glycosylation of membrane proteins and may actually increase the incorporation of fucose and glucosamines into large complex membrane glycoproteins (Figure 1B).

This comparative study of normal and anaplastic cells *in vitro* has shown differences and similarities in the induction of morphologic differentiation by NGF and/or GLU. The real relevance of such a treatment regime to *in vivo* systems remains to be established.

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Chapter 4

The *in vitro* effects of nerve growth factor on the detection of intermediate filaments expressing glial fibrillary acidic protein epitopes by rat anaplastic astrocytoma cells.

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Abstract

Intermediate filament (IF) proteins of neonatal rat astrocytes are shown to express glial fibrillary acidic protein (GFAP) and vimentin epitopes detected by immunocytochemical staining. Stellate astrocytes showed greater staining intensity than epithelioid astrocytes. Nerve growth factor (NGF) (500 *ng*/ml) increased the intensity of GFAP staining by neonatal astrocytes following treatment for 48 hrs. NGF (12.5 *ng*/ml) also induced IFs showing GFAP epitope expression in anaplastic astrocytoma cells. This GFAP expression was fixation-sensitive. Paraformaldehyde fixation preserved GFAP epitopes while alcohol fixation abolished GFAP epitopes expressed by anaplastic astrocytoma cells. GFAP and vimentin IFs were found to be perinuclear, nuclear and nucleolar in distribution. The significance of these findings is that alkylnitrosourea chemical-induced mutagenesis and carcinogenesis of astrocytes may be responsible for changes in IF protein. These changes are suspected to be due to changes in gene structure which may be further characterized by changes in IF protein solubility and expression of GFAP epitopes.

Running Title: NGF induced expression of GFAP by astrocytoma cells.

Key Words: NGF, GFAP, intermediate filaments, astrocytoma cells, immunocytochemistry

Introduction

Intermediate filaments (IFs) along with microtubules and actin microfilaments constitute the three major types of filamentous proteins of cells. IFs are recognized by their intermediate size of 7-11 nm in diameter, which is intermediate to the 4-6 nm actin microfilaments and the 22-25 nm microtubules. IFs are also tissue specific and currently are divided into five major groups, which include glial fibrillary acidic protein (GFAP) and vimentin. Specific IFs can be detected in cell populations by the use of labeled monclonal antibodies. Therefore, knowledge of the specific type of IF expressed by a cell or cell population could result in a determination of the specific cell type.

Astrocytes are known to express two types of IFs, GFAP and vimentin (Eng *et al*, 1971; Bignami and Dahl, 1974; Liem *et al*, 1978). Vimentin is expressed by astrocytes and a variety of mesenchymal cell types such as fibroblasts, macrophages and endothelial cells. Vimentin has also been reported to be preferentially expressed by a variety of cell types maintained in tissue culture (Franke *et al*,1978, 1982; Pateau *et al*, 1979). Vimentin, therefore, is not considered to be an astrocyte-specific IF. Its expression and distribution have been found to decrease during maturation (Pixley *et al*, 1984). It is basically expressed by embryonic non-differentiated astrocytes rather than mature, differentiated astrocytes (Dahl *et al*, 1981). There is also a vimentin-GFAP transition which occurs during astrocyte differentiation. This transition is initiated at the time of myelination (Dahl, 1981; Yokoyama *et al*, 1981). Therefore, vimentin IFs are expected to be found in proliferating populations of astrocytes which in turn are found in greatest numbers in the

periventricular germinal layers and the non-myelinated areas of the white matter in the brain.

GFAP is an astrocyte-specific IF which is expressed by astrocytes from the early post-natal period of development through end-stage differentiation (Bock, 1978; Eng and Bigbee, 1978; Bignami *et al*, 1980; Eng, 1985). GFAP expression is greatest in fibrous astrocytes of the white matter rather than in protoplasmic astrocytes of the gray matter (Raff *et al*,1983). Extensive expression of GFAP also occurs during the process of reactive astrogliosis.

Astrocytes are easily activated by a number of conditions of the brain, including physical and chemical injury, infectious diseases and immunological responses. Astrogliosis and increased expression of GFAP are also found to occur in cases of brain tumors, being most prominent in astrocytomas (Russell and Rubenstein, 1989). However, experimentally produced astrocytomas of the rat induced by methylnitrosourea (MNU) rapidly lose their ability to express GFAP with increased passage when studied in cell culture.

Since the discovery of IFs in the 1960's they have been suspected to be a constituent of the cytoskeleton (Biberfield *et al*, 1965; Sternlieb, 1965; Ishikawa *et al*, 1968). This has been supported by their cellular distribution and their high degree of insolubility under normal physiologic conditions. However, more recent studies have found IFs to be associated with membrane-bound sub-cellular structures and enzyme systems (Lin and Feramisco, 1981; Klymkowsky *et al*, 1983). In addition, IFs have been found to bind to DNA and RNA. These new reports suggest that IFs may not function merely as mechanical integrators of intracellular space, but may be important regulators of nuclear and membrane processes (Lazarides, 1980, 1982; Franke *et al*, 1982; Osborn *et al*, 1982).

Fixation methods have been shown to play important roles in immunocytochemistry (Sternberger, 1979; Polak and VanNoorden, 1983; DeArmond and Eng, 1984). Different fixatives have been implicated in the preferential immunohistochemical-staining of astrocytes for GFAP IFs (Shehab *et al*, 1990). Alcohol fixation caused the preferential staining of GFAP in fibrous astrocytes, whereas paraformaldehyde fixation caused the preferential staining of GFAP in protoplasmic astrocytes. These reports suggest that treatment of astrocytoma cells with different fixatives may affect the detection of GFAP by immunocytochemical staining techniques.

Growth factors which act as differentiation promoters of astrocytes have long been suspected to induce characteristic changes in cytoskeletal proteins. Nerve growth factor (NGF) is a widely recognized differentiation promoter that has been found to induce morphologic differentiation in glioma cells (Marushige *et al*, 1987; Marushige *et al*, 1989). Although the molecular mechanisms by which NGF promotes cell differentiation remain largely unknown; previous studies in our department using different culture and immunostaining techniques have shown that NGF-induced differentiation of astrocytoma cells is accompanied by an extension of cytoskeletal proteins (Marushige *et al*, 1989). In addition, NGF was not found to induce GFAP expression. However, other filaments as well as microtubules were found to gradually extend and fill cytoplasmic processes during morphologic differentiation induced by NGF. In these studies the cytoskeleton was considered to be the major factor responsible for the cell's expression of shape and differentiation. Therefore, variations in cytoskeletal components could give rise to variations in shape, morphologic characteristics and function, since morphology and function of specific cell populations tend to go hand in hand.

The purpose for this study is to determine if NGF induces rat anaplastic astrocytoma cells to express IFs which can be detected by GFAP monoclonal antibodies and to determine if differences in fixatives affect the detection of GFAP IFs by immunocytochemical staining. In addition, this report also addresses IF function and why MNU experimentally-induced astrocytoma cells may or may not express GFAP IFs.

Materials and methods

Materials

NGF (2.5S, grades I and II), anti-vimentin (mouse) and anti-GFAP (mouse) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN,USA). Fetal calf serum (FCS) was purchased from Hyclone Sterile Systems Inc. (Logan, UT, USA). All culture media were prepared using stock solutions, chemicals and supplies from Life Technologies Inc. GIBCO Labs (Grand Island, NE, USA), Corning Glass Works Inc. (Corning, NY, USA) and Sigma Chemical Co. (St. Louis, MO, USA). Hl-1 supplement and concentrate were purchased from Endotronics Inc. (Coon Rapids, MN, USA). Anti-mouse-FITC (goat), mouse serum and L-glutamate were purchased from ICN-Biomedicals Inc. (Costa Mesa, CA, USA).

Neonatal astrocyte cultures

Neonatal rat astrocytes (NR-1) were generated using 4-day-old Fischer rat pups. The brains of four rat pups were removed following euthanasia and placed in a petri dish containing warm DMEM high-glucose media. The meninges were removed and the brain stem and cerebellum separated. The cerebral tissues were minced into small pieces (<0.5 mm), combined and placed into a sterile centrifuge tube containing 5 ml of DMEM high-glucose media. This suspension of brain tissue was then centrifuged at 750 X g for a period of 10 min. The supernatant fluid was removed and discarded and replaced with 1.5 ml of Collagenase II-S (Sigma) solution (0.8%, w/v). The enzyme treatment procedure was followed by incubating the cells on a warm

water bath (37°C) shaker for 30 min, after which the brain tissue was completely dissociated to a cell suspension by intermittently pipetting with a sterile glass pasteur pipette supplied with a cotton filter. At no time during preparation of these cultures were the cells allowed to cool below 30°C. Following the enzyme treatment the cell suspension was centrifuged at 200 X *g* for a period of 10 min and the supernatant fluid removed and discarded. An equal amount (approx. 3 ml) of DMEM high-glucose medium containing 15% FCS was gently layered on top of the cell pellet. Then the upper 2/3 of the cell pellet was resuspended in fresh medium using gentle pipetting action. A 1 ml aliquot of this cell suspension was quickly removed and used to seed primary cultures at approximately a 1:20 split ratio. These stock cultures were started and maintained in 25 cm^2 tissue culture flasks (Corning) containing 5 ml of complete medium. The medium was replaced with fresh medium the following day and replaced every 3 days thereafter. Neonatal astrocytes used in these experiments were at their 3rd passage. The astrocytic character of these cells was indicated at second and third passage not only by morphology and their ability to take up γ-aminobutyric acid, but by their high content of the astrocyte-specific, GFAP. These cells were 95% GFAP positive. In addition, 95% of the cells were epithelioid-like in morphology.

Astrocytoma cells

The rat anaplastic astrocytoma cells were supplied by Dr. A. Koestner of the Department of Pathology, Michigan State University, East Lansing, MI. from his cell storage bank. The T-9 cell line originated from a high grade-anaplastic astrocytoma induced in Fischer rats by treatment with N-methyl-N-nitrosourea (MNU) (Schmidek *et al*, 1971). Stock cultures of $T\text{-}9_a$ and $T\text{-}9_b$ were established in 25 cm^2 tissue culture flasks and maintained in complete

serum-supplemented medium (DMEM and RPMI 1640/Hams F12) with replacement every 3rd day and the splitting of cells every 6th day at a 1:100 split ratio (1-2 X 10^3 cells). The difference in the two T-9 cell populations was that $T\text{-}9_a$ was 20 passages behind $T\text{-}9_b$.

Culture conditions

Cells were cultured in a Hotpack CO_2 Incubator which was maintained at 5% CO_2, 37°C and constant humidity. Both neonatal astrocytes and astrocytoma cells ($T\text{-}9_a$ and $T\text{-}9_b$) were split into multi-well (6 well) culture plates (GIBCO Labs) containing 3 ml of complete medium and a polylysine-coated glass coverslip (1 μg/ml ddH_2O). The complete medium, serum supplemented, was changed the following day and replaced with either of three chemically defined medium (CDM). CDM HL1A was composed of DMEM supplemented with 1% (v/v) HL-1 supplement, 400μM glutamine, gentamycin at 10 μg/ml, glucose at 1 mg/ml, $CaCl_2$ at 175 μg/ml and $MgSO_4$ at 125 μg/ ml. CDM HL1B was also composed of DMEM and contained the same supplementation as HL1A but also contained hydrocortisone at 1.6 μg/ml, prostaglandin F2-alpha at 440 *n*g/ml, putrescine at 78 μg/ml, basic-fibroblastic growth factor at 8.8 *n*g/ml and myelin basic protein at 440 *n*g/ml. This HL1B medium was a slight modification of that originally proposed by Morrison and De Vellis in 1981 as a CDM that initiates differentiation of astrocytes. HL-1 supplement contained 29 μg/ml total protein with 15 μg/ml insulin and contained no additional growth factors or glutamate. CDM HL1C was purchased as a complete CDM. It contained 50μM glutamate and was supplemented with gentamycin at 20 μg/ml and 400μM glutamine.

Cell culture treatment

NR-1, T-9_a and T-9_b cells used in these studies were seeded at 6-12,000 cells/ml into 3 ml of complete, serum-supplemented medium at initial plating. The medium was changed the following day to serum-free CDM. Three days later the CDM was replaced with fresh CDM of the same type and NGF or serum added. NGF grade II was added at 500 *n*g/ml (NR-1 and T-9_a), NGF grade I at 12.5 *n*g/ml (T-9_b) or serum added at 10% (300 μl/3 ml medium). Medium and treatment of T-9_b cells were exchanged for fresh medium and treatment every 12 hrs; this was repeated 4 times. All cell cultures were treated with NGF or serum for 48 hrs prior to cell fixation.

Indirect immunofluorescence of vimentin and GFAP

Intracellular GFAP and vimentin IF antigens were detected in neonatal astrocytes and T-9_a anaplastic astrocytoma cells following *alcohol fixation* in ice-cold ethanol/acetone (1:1; 80%/100%, v/v) for 5 min. GFAP IFs were detected in T-9_b anaplastic astrocytoma cells following *paraformaldehyde fixation* in 2% paraformaldehyde prepared in 100mM cacodylate buffer, pH 7.4. Fixer was removed and cells were immersed in 50% methanol for 3 min followed by 100% methanol for 3 min. Methanol was removed and cells were rinsed in phosphate-buffered saline (PBS) (140mM NaCl, 12mM Na_2HPO_4, 3.5mM NaH_2PO_4), pH 7.2, for 10 min. The cells on coverslips were overlaid with GFAP or vimentin primary monoclonal antibody (100 μl) and incubated for 30 min. The anti-GFAP (mouse) and anti-vimentin (mouse) primary antibodies (20 & 50 μg/ml) were each used at 1:300 dilutions in PBS. The cover slips with cells attached were extensively rinsed 3 times for a total of 20 min with 2 ml PBS, pH 7.2 on a rotary shaker (NR-1 and T-9_a) or by manually swirling (T-9_b). After extensive rinsing the cells were overlaid with FITC-

labeled goat anti-mouse (1:50) secondary antibody (100 μl) and incubated 30 min. These procedures were all carried out in the dark. After incubation with secondary antibody the cover slips were rinsed once in 2 ml of PBS, pH 7.2 for 10 min on an orbital shaker (NR-1 and T-9_a) or by manually swirling (T-9_b). The cells were then overlaid with normal mouse serum (10%) and incubated for 30 min. Cover slips were rinsed extensively, 3 times, in PBS, pH 7.2, for a total of 20 min, on an orbital shaker (NR-1 and T-9_a) or by manually swirling (T-9_b). Cover slips were mounted on glass slides with permamount. Cells were viewed with a Leitz phase contrast microscope fitted with epifluorescent light sources and IFs photomicrographed to show FITC-labeled specificity. Controls which never showed immunofluorescence included substitution with normal mouse serum (10%), omission of primary antibody or elimination of FITC-labeled anti-mouse secondary antibody.

Results

Neonatal astrocytes cultured in HL1B CDM were found to be vimentin-positive by indirect immunocyto-staining following alcohol fixation (Figure 1). Cells cultured in HL1B CDM were found to be GFAP-positive by indirect immunocyto-staining following alcohol fixation (Figure 2A). Cells cultured in CDM and treated with NGF (500 *n*g/ml) were found to increase in fluorescent intensity for GFAP. Intensity was greatest following NGF treatment, in cells cultured in HL1B. In addition, stellate cells were found to show greater intensity for GFAP immunocyto-staining than protoplasmic type cells (Figure 2B).

T-9$_a$ anaplastic astrocytoma cells cultured in HL1A or HL1B CDM were found to be negative for GFAP immunocyto-staining following alcohol fixation. There was no response to NGF treatment detectable by immunocytochemistry. In addition, these cells, cultured under these conditions and fixed in alcohol, were found to show an extremely weak fluorescence to FITC-labeled secondary antibody following exposure to anti-vimentin primary monoclonal antibodies.

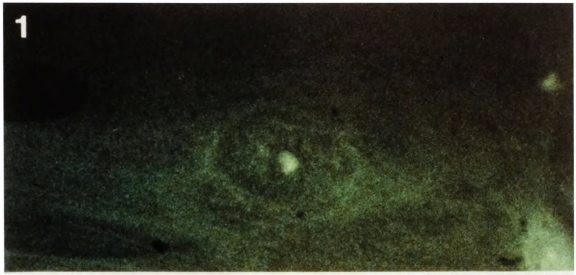
T-9$_b$ anaplastic astrocytoma cells cultured in HL1C CDM were found to be negative for GFAP immunocyto-staining following paraformaldehyde fixation. However, cells cultured in HL1C and treated with NGF (12.5 *n*g/ml)

under different conditions than T-9a anaplastic astrocytoma cells were found to be GFAP positive after fixation in paraformaldehyde and exposure to goat anti-mouse-FITC labeled secondary antibody following exposure to mouse anti-GFAP primary monoclonal antibody (Figure 4).

This treatment with NGF also induced noticeable changes in mophologic characteristics (Figure 3) which were similar to the morphologic changes noted in Chapter 3 (Figure 1B).

Figure 1. Vimentin indirect immunofluorescent staining of neonatal **rat astrocytes.** Cells were cultured in HL1B CDM and ***treated with NGF (500 ng/ml)*** using goat anti-mouse-FITC labeled secondary antibody and mouse anti-vimentin primary antibody following alcohol fixation. Notice the diffuse staining of the cytoplasmic regions and the intense staining of the perinuclear, nuclear and nucleolar regions. X 1225

Figure 2. GFAP indirect immunofluorescent staining of neonatal rat astrocytes . NR-1 cells were cultured in HL1B CDM with ***no treatment*** **(2A)** and ***treated with NGF (500 ng/ml)*** **(2B).** Cells were prepared for GFAP detection using goat anti-mouse-FITC labeled secondary antibody and mouse anti-GFAP primary antibody following alcohol fixation. Notice the intense staining of the perinuclear, nuclear and nucleolar regions (2A) and the intense staining of the fibrous type astrocyte and the lack of intensity to its nucleolar region. The protoplasmic type astrocyte shows diffuse staining of the cytoplasmic regions and greater intensity to the perinuclear, nuclear and nucleolar regions. X 1225

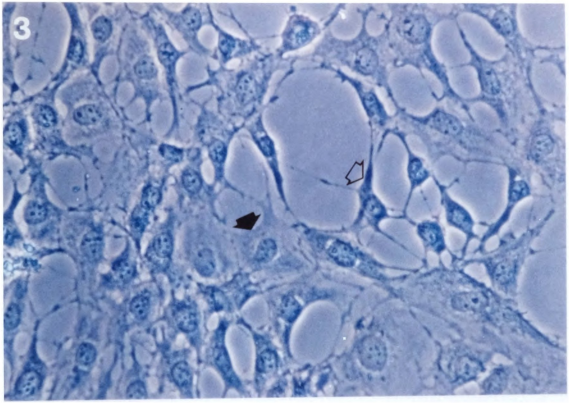

1

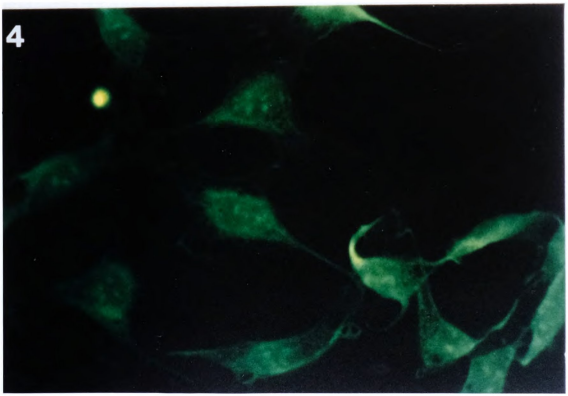

2A


2B

Figure 3. Phase contrast photomicrograph of anaplastic astrocytoma cells. Cells were cultured in HL1C CDM and treated with NGF (12.5 *n*g/ml) every 12 hrs for 48 hrs prior to fixation in paraformaldehyde. Notice the cellular extensions and cytoplasmic processes. Also notice that some cells appear paler and more epithelioid (solid arrowhead) and others appear more dense with condensed cytoplasm being more characteristic of fibrous astrocytes (open arrowhead). X 269

Figure 4. GFAP indirect immunofluorescencent staining of anaplastic astrocytoma cells. Cells were cultured in HL1C CDM and treated with NGF (12,5 *n*g/ml) every 12 hrs for 48 hrs. Goat anti-mouse-FITC labeled secondary antibody and mouse anti-GFAP primary antibody were used following paraformaldehyde fixation. Notice the diffuse staining of the cytoplasm and the intense staining of the perinuclear and nucleolar regions. X 269


3


4

Discussion

GFAP is considered to be the primary intermediate filament of mature differentiated astrocytes (Eng, 1985). Today, GFAP is widely accepted to be an astrocyte-specific intermediate filament (Eng, 1985; Dahl *et al*, 1986). Vimentin, however, does constitute the major intermediate filament of immature astrocytes (Dahl *et al*, 1981; Schnitzer *et al*, 1981). The changes in morphology which accompany cell differentiation in astrocytes have been shown to be coupled to a shift in IFs from vimentin to GFAP, which begins about the time of myelination during development (Dahl, 1981; Yokoama, 1981).

It was reported in previous studies in our department that NGF induces morphologic differentiation in neoplastic astrocytes (Marushige *et al*, 1987, 1989). In these studies, GFAP was not detected in T-9 astrocytoma cells using immunocytochemical staining techniques following alcohol fixation. However, vimentin and GFAP containing IF were detected by IF isolation using a standard technique and western blot analysis.[1] There also was no detection of GFAP in cells even after treatment with NGF (5 μg/ml) using immunocyto-staining, following alcohol fixation (Marushige *et al*, 1989). The data in this study support those findings. That is, GFAP is undetectable in T-9 astrocytoma cells cultured in CDM and fixed in alcohol, even when cells are treated prior to fixation with NGF. However, because fixation methods have been previously shown to play an important role in immunohistocytochemistry (Sternberger, 1979; Polak and VanNoorden, 1983;

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[1] Marushige unpublished personal communication.

DeArmond and Eng, 1984), alcohol and paraformaldehyde fixatives were compared in this study. Previous reports had shown preferential histochemical staining of protoplasmic and fibrous astrocytes in the rat with GFAP monoclonal antibodies, using a variation of fixatives (Shehab *et al*, 1990). The results presented here show that GFAP epitopes can be detected by FITC-labeled secondary antibodies in anaplastic astrocytoma cells, following NGF treatment, using paraformaldehyde fixation (Figure 2). However, T-9_b astrocytoma cells cultured under similar conditions to those earlier reported from our department were found, as reported before, to be negative for GFAP following paraformaldehyde fixation, but without NGF treatment. GFAP epitopes were detected only after treatment with NGF and paraformaldehyde fixation. These results, therefore, indicate that alcohol and paraformaldehyde fixation may have opposite as well as preferential effects on GFAP epitopes expressed by different morphologic types of astrocytes and astrocytoma cells. In addition, it appears that NGF may preferentially increase the expression of IF proteins which share the GFAP epitope common to the GFAP IF protein of protoplasmic astrocytes. This has been further supported by other studies on IFs of neonatal rat astrocytes and astrocytoma cells, which deal with protein soluability and immunochemical detection of epitopes. These additional studies include IF protein isolation using a variation of standard technique and western blots for GFAP, vimentin and protein. These studies suggest that GFAP epitopes may not be preserved in the IFs of anaplastic astrocytoma cells following alcohol fixation and could therefore go undetected. These results further suggest that NGF may have a positive effect on the expression of GFAP epitopes in IFs of anaplastic astrocytoma cell's. Although, quantitative analysis was not carried out.

This report, therefore, supports previous findings which have reported positive and/or negative results on the detection of GFAP by immunohistochemical staining of protoplasmic and fibrous astrocytes in serial brain sections (Shehab *et al*, 1990) and also lends some understanding to conflicting reports on the expression and detection of GFAP by immunocytochemical staining of astrocytes and astrocytoma cells grown and cultured under various conditions and prepared using different methods of detection. More importantly, these results appear to suggest that a unique IF may be expressed by T-9 anaplastic astrocytoma cells which shares a GFAP epitope with neonatal rat GFAP. This GFAP epitope is sensitive to alcohol fixation but preserved by paraformaldehyde fixation, while the IF differs in protein solubility characteristics from the definitive GFAP IF expressed by neonatal rat astrocytes *in vitro* and adult rats *in vivo*.

The hypothesis that a unique IF may be expressed by anaplastic astrocytoma cells which shares a GFAP epitope with IFs expressed by protoplasmic astrocytes, is supported by the numerous reports on MNU induced chemical lesions, involving guanine, which are responsible for the generation of this tumor type (Gercham and Ludlum, 1973; Kleihues and Magee, 1973; Singer, 1975; Kleihues, 1982). In addition, this hypothesis can also be supported by the numerous reports dealing with the expression and function of IFs (Traub, 1983; Traub and Vorgias, 1983, 1984; Vorgias, 1983; Traub, 1985; Miura *et al.*, 1990). The promoter sequence and the transcriptional startpoint and promoter function of the mouse GFAP gene has been characterized (Miura *et al.*, 1990). It has been reported that the *cis* elements for astrocyte specific expression is located within 256 base pairs from the transcription startpoint. Three *trans*-acting factor binding sites have also been defined and identified as GF I, GF II, and GF III. These binding sites are

high in their guanine content. Mutations in GF II have been shown to drastically reduce promoter activity. Base substitutions in GF I and GF III have been shown to abolish cell-specific expression. Since O^6-methylation of guanine is reported to be a major result of MNU exposure, it is suggested that formation of O^6-methylguanine in the GFAP gene could lead to an important mutagenic reaction and affect the expression of GFAP and IFs protein structures. Future studies should be directed to establish whether a definite correlation exists between the level of O^6-methylguanine incorporation and the expression of GFAP IFs or other potential mutagenic events in this experimental brain tumor model.

In summary, GFAP and vimentin IF proteins are shown to be located in perinuclear, nuclear and nucleolar regions of astrocytes (Figure 1, 2A, 2B) and astrocytoma cells (Figure 4). IF proteins expressing GFAP epitopes are induced in astrocytoma cells by NGF. This induction of IF proteins expressing GFAP epitopes by astrocytoma cells may take place through NGF's generation of cyclic AMP. Cell specific gene expression has been speculated to be regulated by a combination of a variety of *trans*-acting factors also containing multiple *cis*-regulatory promoter elements. GF I is a distinct *trans*-acting factor which contains a homologue of the AP-2 binding site (Miura *et al.*, 1990). AP-2 is a transcription factor which mediates transcription activation in response to at least two signal-transduction pathways, protein kinase C activation (phorbol esters) and protein kinase A activation (cyclic AMP) (Imagawa *et al.*, 1987). NGF induced cyclic AMP therefore may act through activation of protein kinase A or possibly independently and susequently activate the GFAP promoter region resulting in increased expression of GFAP IFs. The GFAP epitope is further suggested to be preserved by paraformaldehyde fixation and abolished by alcohol fixation. IFs of

astrocytoma cells expressing this GFAP epitope may also show different characteristics in IF protein solubility which has been suggested by the variation in IF protein isolation between neonatal astrocytes and anaplastic astrocytoma cells. This variation in IF protein may result from secondary mutational events occurring to guanine nucleotides in the GFAP and vimentin IF gene, which has an abundant guanine content. IFs have been shown in this study to have a perinuclear location in the cell. The significance of IF proteins having a nuclear function may be acknowledged in future studies by exhibiting their involvement in regulation of mitogenesis, gene expression and nuclear rRNA transport.

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Chapter 5

An *in vitro* Study on the Effect of Nerve Growth Factor on Gamma-aminobutyric Acid Uptake by Neonatal Rat Astrocytes and Rat T-9 Astrocytoma Cells

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Abstract

The effect of nerve growth factor (NGF) on the *in vitro* uptake of gamma-aminobutyric acid (GABA) by rat astrocytes is reported. NGF's effect on GABA uptake by neonatal rat astrocytes is compared to NGF's effect on GABA uptake by rat anaplastic astrocytoma cells. Both of these cell types originate from the Fischer rat genetic pool. NGF is found not to significantly increase GABA uptake by neonatal astrocytes or anaplastic astrocytoma cells cultured in chemically defined media. The experimental uptake data presented here further show that NGF actually acts to decrease GABA uptake by neonatal rat astrocytes. We suspect that NGF causes this effect by somehow affecting the $GABA_A$ receptor. Seizures induced as a consequence of decreased synaptic levels of GABA is well accepted. If NGF were to increase GABA uptake, it could be expected to potentiate seizures. However, the present study does not support this hypothesis. This report, therefore, concerns the potential use of NGF as a pharmacologic agent for the differentiation and arrest of tumor growth as well as for the reduction of seizures.

Running Title: The effect of NGF on GABA uptake

Key Words: GABA Uptake, nerve growth factor, basic-Fibroblast growth factor, GABA receptors, Neonatal astrocytes, Astrocytoma cells.

Introduction

Gamma-aminobutyric acid (GABA) is widely recognized to be an important inhibitory amino acid neurotransmitter of the CNS (Krnjevic,1974). GABA is inactivated after its release from nerve endings by uptake processes shared by some neurons and glia (Henn and Hamberger, 1971; Iversen & Kelly, 1975). These uptake processes are very similar but can be distinguished by their structural requirements (Bowery *et al.*, 1974). Neuronal and glial uptake of synaptic GABA accounts for 70% of total brain concentrations (Beart, 1976).

Glial cells were first shown to be involved in terminating the action of GABA in autoradiographic studies using tritiated GABA (Hokfelt & Ljungdahl, 1970). High affinity, energy-dependent GABA uptake was soon reported in bulk-isolated astroglial cells (Henn & Hamberger, 1971; Schousboe *et al.*, 1977, 1978). This high affinity uptake was further shown to be sodium-dependent and optimal at physiologic potassium concentrations (Sellstrom & Hamberger, 1975; Schousboe, 1981).

GABA uptake in astrocytes is coupled to the internal movement of one atom of sodium (neuronal uptake is coupled to 2 and 3 atoms) and the external movement of one atom of potassium (Hertz *et al.*, 1978; Martin, 1976; Schousboe, 1981). This implies that GABA uptake is a function of changes in membrane potential and the sodium gradient (Sellstrom and Hamberger, 1977; Larsson *et al*, 1986). In fact, high concentrations of extracellular potassium, upon depolarization, initiate the release of accumulated GABA by neurons in a calcium-dependent fashion (Sellstrom and Hamberger, 1977).

GABA produces its effects as an inhibitory neurotransmitter by enhancing the flux of chloride ions across the cell membrane (Krnjevic, 1974). There are two types of GABA receptors. $GABA_A$ is the major inhibitory receptor and operates a chloride channel. In fact, there are two major types of chloride channels, those that are GABA operated and those that are voltage dependent. One type is active, one type is passive, respectively. This voltage-dependent chloride channel plays an important role in determining the resting membrane potential as well as in maintaining intracellular pH. The $GABA_B$ receptor regulates Cyclic AMP production in concert with other stimulatory receptor-ligand complexes (Enna & Karbon, 1987). Both GABA receptors are vital to a normal functioning nervous system. The inhibition of $GABA_A$ receptors has become the molecular target for the development of many anxiolytic drugs like the benzodiazepines, *e.g.* Valium (Abalis *et al.*, 1983; Fischer & Olsen, 1986). In addition, $GABA_A$ receptors have been found to be the neurotoxic target of many potentially toxic compounds including the chlorinated hydrocarbons and the pyrethroids (Casida & Lawrence, 1985; Abalis *et al.*, 1985; Stark *et al.*, 1986; Gant *et al.*, 1987). Defects in chloride permeability relating to imperfect GABA receptors has also been shown in the hereditary diseases of myotonia and cystic fibrosis (Bryant & Morales-Aguilera, 1971; Frizzell *et al.*, 1986).

Nerve growth factor (NGF) is recognized as a potent differentiation and maturation promoter (Levi-Montalcini & Angeletti, 1968; Greene and Tischler, 1976; Greene & Shooter, 1980; Yankner & Shooter, 1982; Levi-Montalcini, 1987; Marushige *et al.*, 1987) as well as an important factor in the maintenance of selected neuronal cell populations of the nervous system (Thonen *et al.*, 1987; Whittemore & Seiger, 1987; Springer, 1988). Among the studies on astrocyte morphology are reports that morphology influences

GABA uptake (Wilkin *et al.*, 1983). It has been reported that stellate-appearing rat cerebellar astrocytes preferentially take up and concentrate GABA (Wilkin *et al.*, 1983; Levi *et al.*, 1986). It is hypothesized that NGF treatment of anaplastic astrocytomas will result in morphologic differentiation and in an increased uptake of the inhibitory amino acid neurotransmitter GABA.

As a result of NGF's dramatic ability to promote differentiation, it has been suggested as a potential therapeutic agent in the treatment of brain tumors. However, if one assumes that previous reports indicating that increased morphologic differentiation by astrocytes also increases net GABA uptake, then NGF's possible therapeutic benefit would be in question, since increased uptake of GABA would potentially increase the incidence of seizures. Therefore, the purpose of this study was to determine if NGF increases the uptake of GABA. [^{3}H]-GABA uptake by neonatal rat astrocytes and T-9 rat astrocytoma cells was compared under identical *in vitro* conditions using two different chemically defined media.

Materials and Methods

Materials

NGF (2.5 S, grade II) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN,USA). Fetal calf serum (FCS) was purchased from Hyclone Sterile Systems Inc. (Logan, UT, USA). All culture media were prepared using stock solutions, chemicals and supplies from Life Technologies, Inc,GIBCO Labs (Grand Island, NE, USA), Corning Glass Works Inc. (Corning, NY, USA) and Sigma Chemical Co. (St. Louis, MO, USA). Hl-1 supplement was purchased from Endotronics Inc. (Coon Rapids, MN, USA). GABA was purchased from ICN-Biomedicals Inc. (Costa Mesa, CA, USA) and [^{3}H]-GABA (26.9 Ci/mmol) from New England Nuclear, Dupont/NEN Products. (Boston, MA, USA).

Neonatal Astrocyte Cultures

Neonatal rat astrocytes were generated using 4 day old Fischer rat pups. The brains of four rat pups were removed following euthanasia and placed in a petri dish containing warm DMEM high glucose media. The meninges were removed and the brainstem and cerebellum separated. The cerebral tissues were minced into small pieces (<0.5 mm), combined and placed into a sterile centrifuge tube containing 5 ml of DMEM high glucose media. This suspension of brain tissue was then centrifuged at 750 X g for a period of 10 mins. The upper supernatant fluid was removed and discarded and replaced with 1.5 ml of Collagenase II-S (Sigma) solution (0.8%, w/v). The enzyme treatment procedure was followed by incubation of the cell mixture on a

warm water bath (37°C) shaker for 30 mins. The brain tissue was completely dissociated to a cell suspension by intermittently pipetting with a sterile glass pasteur pipette supplied with a cotton filter. At no time during preparation of these cultures were the cells allowed to cool down below 30°C. Following the enzyme treatment the cell suspension was centrifuged at 200 X *g* for a period of 10 mins and the supernatant fluid removed and discarded. An equal amount (approx. 3 ml) of complete culture medium containing 15% FCS was gently layered on top of the cell pellet. Then the upper 2/3 of the cell pellet was resuspended in fresh medium using gentle pipetting action. A 1 ml aliquot of this cell suspension was then quickly removed and used to seed primary cultures at approximately a 1:20 split ratio. These stock cultures were started and maintained in 25 cm^2 tissue culture flasks (Corning) containing 5 ml of complete medium. The medium was replaced with fresh medium the following day and replaced every 3 days thereafter. At the time of second passage medium was changed replacing DMEM with RPMI 1640/Hams F12 (1:1, v/v). Neonatal astrocytes used in the GABA uptake experiments were at their 3rd passage. The astrocytic character of these cells was indicated at second and third passage not only by morphology but by their content of the astrocyte specific, glial fibrillary acidic protein.

Astrocytoma Cells

The rat anaplastic astrocytoma cells were supplied by the Department of Pathology, Michigan State University, East Lansing, MI. from their cell storage bank. The T-9 cell line originated from a high grade anaplastic astrocytoma induced in Fischer rats by treatment with N-methyl-N-nitrosourea (MNU) (Schmidek, *et al.* 1971). Stock cultures were established in 25 cm^2 tissue culture flasks and maintained by complete serum supplemented medium

(DMEM and RPMI 1640/Hams F12) replacement every 3rd day and the splitting of cells every 6th day at a 1:100 split ratio.

Culture Conditions

Cells were cultured in a Hotpack CO_2 Incubator which was maintained at 5% CO_2, 37°C and constant humidity. Both neonatal astrocytes and astrocytoma cells used in the GABA uptake studies were split into multi-well (6 well) culture plates (GIBCO Labs) containing 3 ml of complete medium. This medium helped to assure cell attachment. The complete medium, serum supplemented, was changed the following day and replaced with chemically defined medium (CDM). CDM HL1A was composed of DMEM supplemented with 1% (v/v) HL-1 supplement, 400μM glutamine, gentamycin at 10 μg/ml, glucose at 1 mg/ml, $CaCl_2$ at 175 μg/ml and $MgSO_4$ at 125 μg/ ml. CDM HL1B was also composed of DMEM and contained the same supplementation as HL1A but also contained Hydrocortisone at 1.6 μg/ml, Prostaglandin F2-alpha at 440 *n*g/ml, Putrescine at 78 μg/ml, beta-Fibroblastic Growth Factor at 8.8 *n*g/ml and Meylin Basic Protein at 440 *n*g/ml. This HL1B medium was a slight modification of that originally proposed by Morrison and De Vellis as a CDM that initiates differentiation of astrocytes. HL-1 supplement contains 29 μg/ml total protein with 15 μg/ml insulin and no additional growth factors, glutamate or GABA.

GABA Uptake Experiments

Neonatal astrocytes and astrocytoma cells used in these studies were seeded at 6-12,000 cells/ml into 3 ml of complete, serum-supplemented, media at initial plating. The medium was changed the following day to serum free CDM. Three days later the CDM was replaced with fresh CDM of

the same type and NGF added. NGF was added at 500 *n*g/ml or serum added at 10% (300 μl/3 ml medium). Cells were incubated in NGF or growth factor containing serum for 48 hrs prior to the addition of GABA. Each GABA concentration that was studied (2.5 and 25μmol) contained 3 μCi of [^{3}H]-GABA per 3 ml of culture medium per petri dish. [^{3}H]-GABA had a specific activity of 1 μCi/40 *p*mol. GABA specific activity was established at 1μCi/2.5-25μmol. The standard incubation period for GABA uptake was set at room temperature (RT) (20-25^{o}C) for 10 min. During the uptake incubation, cell culture plates were placed on a Lab-Line rotary orbit shaker (60 rpm) to assure dispersion of GABA. The incubation was stopped by removing the [^{3}H]-GABA containing medium and rinsing the cultures twice in a HEPES buffered saline solution, pH 7.4, which contained the same concentration of unlabeled GABA. Each rinse was for a period of 10 min and was also carried out on a rotary shaker. Following this rinse procedure the cells were extracted by placing 2 ml of a freshly prepared solution of 1M perchloric acid into each well. Cells were extracted for 10 min, again utilizing the rotary shaker. Cells were then given a post-extraction rinse using 2 ml of 0.1M Tris buffer, pH 7.4. This rinse solution was combined with the extract solution. Throughout this procedure cells remained attached to the culture plate. GABA uptake data were recorded as counts per minute (cpm) and then normalized using specific activity of GABA and cpm/protein concentration and reported as *n*mol GABA/mg protein/time (10 min). The experiment was designed to compare GABA uptake by neonatal rat astrocytes and rat anaplastic glioma cells in two different CDM and to determine the effects of NGF, serum and hypothermia (Table 1). Three or more replicate cultures were analyzed for each study.

Scintillation Analysis

The procedure was carried out using a Packard 300 scintillation counter. Uptake extract solution (0.8 ml) was added to Aquasol II scintillation fluid (10 ml).

Protein Concentration

The Bio-Rad method of protein analysis was used. Extract uptake solution (0.8 ml) was combined with 200 μl of reagent and absorbance checked at 595 nm. This was compared to a standard curve using Bovine IgG and quantitated accordingly. The average protein concentration for T-9 cultures was 36.8 μg/ml. and 38.8 μg/ml for NR-1 cultures.

Statistical Analysis

Determination of statistically significant differences between experimental groups was formed utilizing the Students-t test. Differences were considered to be significant when p values = or <0.05 were obtained for n= or >3. Standard error of the means (SEM) was also determined and reported where significance was indicated.

Results

Effects of Medium on GABA Uptake

The net uptake of GABA (2.5 and 25μmol) by NR-1 and T-9 cells in HL1A and HL1B CDM is compared. Cells were cultured in both media and incubated with GABA for 10 min. There was a significant difference in GABA uptake by these cells in these different media (Table 1.). GABA uptake was much greater by cells cultured in HL1A.than in HL1B.

Effects of NGF on GABA Uptake

This study examines the effect of NGF on GABA uptake (2.5 and 25μmol) following a 10 min incubation at room temperature by NR-1 and T-9 cells cultured in HL1A and HL1B CDM. NGF (500 ng/ml) was found to significantly decrease the uptake of GABA (2.5 and 25μmol) by NR-1 astrocytes cultured in HL1B CDM (Table 1.).

Effects of Fetal Calf Serum on GABA Uptake

This study determined the effects of serum (100 μl/ml medium) on GABA uptake following a 10 min incubation at room temperature by NR-1 and T-9 cells cultured in HL1A CDM. The addition of fetal calf serum (FCS) in place of NGF was not found to significantly increase the uptake of GABA (25μmol) by T-9 astrocytoma cells nor by NR-1 astrocytes (Table 1).

Control Study on the Effects of Hypothermia on GABA Uptake.

GABA uptake by NR-1 and T-9 cells was determined at temperatures <4°C. Cell cultures were placed on ice for 90 min prior to a 10 min GABA incubation at temperature <4°C. Most cellular uptake processes are generally stopped by low temperatures and recover when temperature is increased. Hypothermia was found to cause a significant decrease in the uptake of GABA (25μmol) by both NR-1 astrocytes as well as T-9 astrocytoma cells (Table 1.). This suggests that oxidative phosphorylation and the subsequent generation of ATP is a necessary component for GABA uptake by both cell types.

TABLE 1. *GABA uptake by neonatal astrocytes and anaplastic astrocytoma cells cultured under varying conditions and treatments.*

Culture Conditions and Treatment (concentration, temp, medium,treatment)	GABA Uptake by Cell Type (nmol GABA/mg protein/10 min)	
	NR-1	**T-9**
25μmol GABA, RT, HL1A	483.0 (62.5)	571.0 (45.5)
2.5μmol GABA, RT, HL1A	34.0 (1.0)	39.7 (3.2)
25μmol GABA, RT, HL1B	170.3 (22.7)	311.0 (4.0)
2.5μmol GABA, RT, HL1B	15.7 (0.3)	36.33 (6.2)
25μmol GABA, RT, HL1A, NGF	349.3 (4.7)	494.7 (20.5)
2.5μmol GABA, RT, HL1A, NGF	30.5 (1.5)	41.7 (0.8)
25μmol GABA, RT, HL1B, NGF	122.3 (7.7)	329.7 (11.7)
2.5μmol GABA, RT, HL1B, NGF	11.3 (0.3)	32.7 (2.2)
25μmol GABA, RT, HL1A, SERUM	398.0 (1.2)	780.7 (67.6)
25μmol GABA, 4°C, HL1A	101.7 (6.2)	307.0 (13.1)

Statistically significant differences in GABA uptake are shown between NR-1 and T-9 cells cultured in HL1B medium, between HL1A and HL1B medium in both NR-1 and T-9 cells, between NGF treated NR-1 cells and non-treated cells cultured in HL1B medium at 2.5 and 25μmol GABA and in both cell types in response to cold temperature. All comparisons were made using the Students-t test with $p<0.05$. Standard error of the mean (SEM) is given in brackets.

Discussion

The purpose for this study was to determine if NGF has any significant effect on GABA uptake. The reason relates to NGF's reported ability to initiate morphologic differentiation (Greene & Tischler, 1976; Marushige *et al.*, 1989) as well as its reported ability to arrest growth and proliferation (Marushige *et al.*, 1987). These reports, in addition to many other similar reports in the literature, suggest that NGF may have benefit in the treatment of tumors of the nervous system, particularly treatment of tumors of the glial type. There are other reports in the literature that have suggested that growth factors, hormones and other chemicals that promote differentiation may also increase the uptake of the inhibitory neurotransmitter GABA (Wilkin *et al*, 1983). There are still other reports which state that NGF increases amino acid transport, including utilization of the gamma-glutamyl cycle (Yankner and Shooter, 1982). This could be interpreted to mean that NGF, like other differentiation promoters, could increase the net uptake of GABA. If NGF were to increase GABA uptake, it's supplementation to specific areas of the CNS would be expected to potentially induce seizures. The proposition that seizures arise as a consequence of GABA deficiency was suggested years ago (Iwama & Jasper, 1957; Elliot & Jasper, 1959). It has now become universally accepted that seizure activity and a lowered GABA activity go hand-in-hand (Krnjevic, 1983) and a net increase in GABA uptake could be expected to bring about a net decrease in synaptic GABA concentrations. Therefore, if NGF were to have any hopes of development as a pharmacologic agent in the

treatment of tumors of the CNS, it must first be shown that NGF does not have any detrimental effects on GABA's normal uptake process

Because of the importance of how NGF might affect tumor cells differently than it does normal cells, this study draws a comparative picture of the differences in cell type. The study compares two cell types that evolved genetically identical, both originated from identical lines of Fischer rats, except that one cell type was transformed genetically by exposure to N-methyl-N-nitrosourea (MNU) (Schmidek, *et al.*, 1971; Swenberg *et al.*, 1972). The rat anaplastic astrocytoma (T-9) cell line is well-established and has been characterized very well (Ko *et al.*, 1980). The neonatal rat astrocytes (NR-1) were isolated and cultured specifically for this study. The culture methods of NR-1 and T-9 were made to be as identical as possible.

Medium preparation is a very important factor in any study where morphology is an intricate part of the expected outcome. Different medium preparations have been shown to initiate differentiation (Morrison & DeVellis, 1981). In this experimental model, the differentiating medium (HL1B) brought about a significant decrease in the uptake of GABA. This suggests that one of the factors present in the HL1B medium may preferentially decrease the uptake of GABA. This decrease could be brought about by one of the constituents of the HL1B medium interfering with GABA binding or by initiating a GABA release mechanism. We could speculate that this action is somehow related to basic fibroblast growth factor (basic-FGF). Little is known of the membrane receptor-induced activities of basic-FGF. It has been reported to be mitogenic in the induction of endothelial growth (Thomas *et al.*, 1985; Bohlen *et al.*, 1985; Lobb *et al.*, 1985), but has not been proven to be mitogenic in astrocytes (Leutz and Schachner, 1981). It has also been found to be a neurotrophic factor increasing the survival of neurons and

growth of neurites in culture, similar to NGF (Morrison, *et al.*, 1986, 1988; Unsicker *et al.*, 1987; Walicke *et al.*, 1986). Therefore, FGF, like NGF, may be more important in the preservation and maintenance of astrocytes cultured under these conditions. Hl1B medium was found to act synergistically with NGF in reducing GABA uptake by NR-1 cells. This is shown in Table 1. Glycoprotein molecules like heparin have been shown to be necessary for high-affinity binding of basic-FGF to its receptor (Yayon *et al.*, 1991). This further implies that imperfect glycosylation of membrane protein constituents of the FGF receptor complex may also be an important factor in creating differences in the cellular responses of NR-1 and T-9 cells to HL1B CDM.

NGF causes a significant reduction in the uptake of GABA by NR-1 astrocytes when cells are cultured in HL1B medium and treated with NGF for 48 hrs prior to uptake (Table 1.). The data reported here show that NGF does not increase the uptake of GABA as other differentiation promoters and conditions favoring morphologic differentiation have been reported to do (Wilkin *et al.*, 1983; Levi *et al.*, 1986)

Although GABA uptake is not significantly changed in T-9 astrocytoma cells, the uptake pattern of GABA in response to NGF treatment suggests a very heterogeneous population of NGF receptor complex. In the study of NR-1 neonatal astrocytes, NGF consistently decreased the uptake of GABA. While in the study of GABA uptake by T-9 astrocytoma cells in the presence of NGF, GABA uptake showed no statistically significant change overall (Table 1). It is speculated that NGF may bring about its action relative to the decrease in GABA uptake by phosphorylation of a GABA binding protein or a structural change in the binding protein induced by the phosphorylation of the NGF receptor resulting in the subsequent release or inhibition of GABA

binding. These speculations may be supported by the data showing an increased uptake of GABA by T-9 astrocytoma cells in the presence of serum (Table 1.). Fetal calf serum is known to contain growth factors whose receptors are associated with tyrosine kinase activity. At least ten oncogene products expressed by different tumor cell types are also associated with tyrosine kinase activity. The availability of high energy phosphate could explain the over activation of other tyrosine kinase processes. Another possibility could be that serum induces growth and the expression of NGF binding sites that are not responsive to tyrosine kinase activation. In either case, NGF has the ability to modulate the uptake of GABA by NR-1 neonatal astrocytes cultured in HL1B medium and NR-1 neonatal astrocytes differ markedly from T-9 anaplastic astrocytoma cells in their response to NGF.

GABA uptake is well-established to be an active uptake process associated with ionic movements which are in turn dependent on the generation of high energy phosphate bonds in the form of ATP. In order to test the GABA uptake experimental model presented here with a control study, GABA uptake was recorded under conditions of hypothermia (temp <4°C). NR-1 neonatal astrocytes showed over a 75% reduction in the uptake of GABA and T-9 anaplastic astrocytoma cells showed over a 50% reduction in uptake of GABA under hypothermic conditions. These data confirm that this experimental model testing GABA uptake responds as expected to conditions of hypothermia.

In summary, NGF has been shown to cause a significant decrease in GABA uptake by NR-1 neonatal astrocytes cultured in a chemically defined medium. This decrease in GABA uptake may be due to activation of NGF receptor-linked kinase and subsequent phosphorylation, resulting in activation of a GABA release mechanism or in the ability of GABA to bind to

$GABA_A$ receptors. This loss in affinity for $GABA_A$ receptors by GABA may in-turn increase activation of $GABA_B$ receptors leading to greater differentiation by increasing intracellular levels of Cyclic AMP.

This study introduces a new procedure for studying uptake by astrocytes and astrocytoma cells. GABA uptake has been determined in cells which were not disturbed prior to initiation of uptake. The determination of cellular uptake was done by recording [^{3}H]-GABA uptake by these cells using analyses of cell extract. GABA uptake is reported in relation to protein extracted. It is known that protein per cell is greater in NR-1 neonatal astrocytes as compared to T-9 anaplastic astrocytoma cells and our results agree. However, this procedure is new and quite different than other published procedures. Therefore, caution should be used in making comparisons of these results to the results of previous uptake studies.

The importance of this study is that NGF does not significantly increase the net uptake of GABA by astrocytes. This means that NGF and other differentiation promoters could be expected to influence GABA uptake independently. These results further show that NGF may potentially decrease seizures by decreasing GABA uptake and therefore could have promise for future therapeutic use as a promoter of tumor differentiation as well as potential use as an anticonvulsant.

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Chapter 6

An *in vitro* Study on Glutamate Uptake by Neonatal Rat Astrocytes and Rat Astrocytoma Cells

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Abstract

This study reports on glutamate (GLU) uptake by neonatal rat astrocytes and rat anaplastic astrocytoma cells cultured in insulin-containing chemically defined media (CDM). Nerve growth factor (NGF) is reported to have no significant effect on GLU uptake by these cells in CDM. However, GLU uptake by astrocytes was significantly lower as compared to the astrocytoma cells when cells were cultured in insulin-containing CDM. The effects of serum and cold temperature on GLU uptake by these cell types, is also reported. In addition, GLU uptake by rat astrocytoma cells was found to be an active uptake process. The potentiation of epileptic-type seizures as well as the production of neuronal cell death as a consequence of increased levels of synaptic GLU are well accepted. The experimental data presented here shows that NGF did not significantly change GLU uptake by neonatal rat astrocytes or astrocytoma cells. These results suggest that NGF may be useful as a pharmacologic agent for the treatment of brain tumors without potentiating an increase of seizures and neuronal cell death.

Running Title: Studies on glutamate uptake by astrocytes.

Key Words: GLU-receptors, GLU-uptake, insulin, nerve growth factor, basic-fibroblast growth factor, neonatal astrocytes, astrocytoma cells, epilepsy.

Introduction

Glutamate (GLU) is widely recognized as the major excitatory amino acid neurotransmitter of the central nervous system (CNS). It is now generally accepted that high levels of GLU are responsible to some degree for producing seizures and mediating epileptic brain damage and neuronal cell death (Sloviter *et al.*, 1985; Sloviter, 1986).

The uptake systems of astrocytes provide a means of clearing both GLU and potassium ions (K^+) from the synaptic space following their release by pre-synaptic and post-synaptic neurons (Schousboe *et al.*, 1981; Schousboe, 1981). The high affinity GLU uptake system is sodium dependent. Sodium and chloride mediate uptake without activation of a homoexchange mechanism (Henn *et al.*, 1974; Schousboe *et al.*, 1977; Hertz *et al.*, 1978; Waniewski and Martin, 1986). Uptake is not dependent on oxidative phosphorylation (Waniewski and Martin, 1986). The uptake of GLU is of particular importance because over-stimulation or persistent stimulation of neurons through inappropriate release or failure to clear this neurotransmitter results in the degeneration and death of certain neurons (Olney, 1969; Rothman, 1984; Simon *et al.*, 1984; Wieloch, 1985; Choi, 1988; Faden *et al.*, 1989).

At least five different types of GLU receptors have been found to exist in the brain. Many of these receptors are shared by neurons and glia (Hosli *et al.*, 1979). These include ionotropic receptors which are coupled to the opening of ion channels in the cell membrane. The ionotropic receptors are identified by their preferential activation by various analogs as N-methyl-D-

aspartate (NMDA) receptor, quisqualate (Quis) receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, kainic acid (KA) receptor and 2-amino-4-phosphonobutyrate (AP4) (Fagg *et al.*, 1986; Johnson and Koerner, 1988). In addition, a GLU receptor has been reported to be associated with the hydrolysis of membrane phosphoinositides (Nicoletti *et al.*, 1986_a, 1986_b, 1987). This receptor functions in membrane signal transduction. Activation of this receptor generates 1,4,5-inositol triphosphate (IP_3) and calcium (Ca^{++}) as second messengers. This second messenger-coupled receptor has been shown to be activated not only by GLU but also by ibotenate and Quis. This receptor is not however thought to be involved in the generation of neurotoxicity induced by GLU. Astrocytes are believed to share only Quis and KA ionotropic receptors as well as the IP3 generating receptor with neurons (Kettenman and Schachner, 1985; Pearce *et al.*, 1987).

Experimentally, GLU has been shown to produce acute glial and dendritic swelling and neuronal necrosis following brain treatment (Sloviter and Dempster, 1985). However, this is in contrast to earlier reports which supported GLU supplementation not only for improved mental behavior but for neurologic conditions of epilepsy and mental retardation (Fonnum, 1984). These earlier reports were supported by GLU's function in the detoxification of ammonia in the brain (Wiel-Malherbe, 1950). Their is no question that glutamate functions not only as a neurotransmitter but as an important constituent of brain metabolism. GLU is consequently thought to be important in the development of cognitive function and in the maintenance of mental health. GLU has proven to be particularly important as an excitatory neurotransmitter transmitter in cortical and hippocampal regions of the brain (Fonnum, 1984).

Insulin is a hormone which serves many functions important to the maintenance and survival of most mammalian cell systems. Insulin has been shown to promote the survival and differentiation of fetal neuronal cells in culture (Hooghe-Peters *et al.*, 1981). However, insulin's most recognized function is the facilitation of glucose transport.

Insulin does not cross the blood brain barrier. In the brain, glucose crosses the blood brain barrier by means of a carrier-mediated, non-energy-requiring facilitated transport. The brain normally maintains a glucose concentration that is twenty times higher than the concentration necessary to saturate hexokinase, the first enzyme of the glycolytic pathway. However, conditions of hypoglycemia could lower the saturation of the glucose carrier and become the rate-limiting factor for the generation of energy. In addition to insulin's role in glucose transport it has been found to affect the amino acid neurotransmitters. Insulin stimulates the sodium-dependent uptake of the inhibitory amino acid neurotransmitter, γ-aminobutyric acid (GABA), into synaptosomes (Herschman, 1986). Insulin treatment has also been shown to dramatically reduce cortical and plasma levels of glutamate in animal studies (Bernasconi *et al.*, 1988). Glutamate content is reduced 50% and 90%, respectively, following insulin treatment. In addition several cell types, with the exception of brain cells, have shown an insulin-dependent depression of insulin receptor levels (Van Schravendijk *et al.*, 1984).

Basic-fibroblast growth factor (basic-FGF) has been found to be plentiful in the central nervous system. Little is known of the membrane receptor-induced activities of basic-FGF on astrocytes or astrocytoma cells. It has been reported to be mitogenic in the induction of endothelial growth (Bohlen *et al.*, 1985; Lobb *et al.*, 1985; Thomas *et al.*, 1985), but has not been proven to be mitogenic in astrocytes (Leutz and Schachner, 1981). It has also been found to

be a neurotrophic factor increasing the survival of neurons and growth of neurites in culture (Morrison *et al.*, 1986; Walicke *et al.*, 1986; Unsicker *et al.*, 1987; Morrison *et al.*, 1988). Therefore, like nerve growth factor, it may be more important in the preservation and maintenance of astrocytes cultured under these conditions.

Nerve growth factor (NGF) is recognized as a potent differentiation and maturation promoter of various neuronal cell types (Levi-Montalcini and Angeletti, 1968; Greene and Tischler, 1976; Greene and Shooter, 1980; Yankner and Shooter, 1982; Levi-Montalcini, 1987; Marushige *et al*, 1987) as well as an important factor in the maintenance of selected neuronal cell populations of the CNS (Thonen *et al.*, 1987; Whittemore and Seiger, 1987; Springer, 1988). As a result of NGF's dramatic ability to promote differentiation, it has been suggested as a potential therapeutic agent in the treatment of brain tumors. Previous reports indicate that increased morphologic differentiation of astrocytes may also increase neurotransmitter uptake (Wilkin *et al.*, 1983); therefore, NGF may have therapeutic benefit since increased net uptake of GLU could potentially decrease the incidence of seizures. Based on these considerations, the purpose of this study was to determine whether NGF increases the net uptake of GLU.

Materials and Methods

Materials

NGF (2.5S, grade II) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN,USA). Fetal calf serum (FCS) was purchased from Hyclone Sterile Systems Inc. (Logan, UT, USA). All culture media were prepared using stock solutions, chemicals and supplies from Life Technologies, Inc,GIBCO Labs (Grand Island, NE, USA), Corning Glass Works Inc. (Corning, NY, USA) and Sigma Chemical Co. (St. Louis, MO, USA). Hl-1 supplement was purchased from Endotronics Inc. (Coon Rapids, MN, USA). Glutamate was purchased from ICN-Biomedicals Inc. (Costa Mesa, CA, USA) and [^{3}H]-GLU (25 Ci/mmol) from New England Nuclear, Dupont/NEN Products. (Boston, MA, USA).

Neonatal Astrocyte Cultures

Neonatal rat astrocytes were generated using 4 day old Fischer rat pups. The brains of four rat pups were removed following euthanasia and placed in a petri dish containing warm DMEM high glucose media. The meninges were removed and the brain stem and cerebellum separated. The cerebral tissues were minced into small pieces (<0.5 mm), combined and placed into a sterile centrifuge tube containing 5 ml of DMEM high glucose media. This suspension of brain tissue was then centrifuged at 750 X g for a period of 10 min. The supernatant fluid was removed and discarded and replaced with 1.5 ml of Collagenase II-S (Sigma) solution (0.8%, w/v). The enzyme treatment procedure was followed by incubation of the cell mixture on a warm water

bath (37°C) shaker for 30 min, after which the brain tissue was completely dissociated to a cell suspension by intermittently pipetting with a sterile glass pasteur pipette supplied with a cotton filter. At no time during preparation of these cultures were the cells allowed to cool below 30°C. Following the enzyme treatment the cell suspension was centrifuged at 200 X *g* for a period of 10 min and the supernatant fluid removed and discarded. An equal amount (approx. 3 ml) of DMEM high glucose medium containing 15% FCS was gently layered on top of the cell pellet. Then the upper 2/3 of the cell pellet was resuspended in fresh medium using gentle pipetting action. A 1 ml aliquot of this cell suspension was quickly removed and used to seed primary cultures at approximately a 1:20 split ratio. These stock cultures were started and maintained in 25 cm^2 tissue culture flasks (Corning) containing 5 ml of complete medium. The medium was replaced with fresh nutrient medium the following day and replaced every 3 days thereafter. Neonatal astrocytes used in the GLU uptake experiments were at their 3rd passage. The astrocytic character of these cells was indicated at second and third passage not only by morphology,and their ability to take up γ-aminobutyric acid, but by their content of the astrocyte-specific, glial fibrillary acidic protein.

Astrocytoma Cells

The rat anaplastic astrocytoma cells were supplied by Dr. A. Koestner of the Department of Pathology, Michigan State University, East Lansing, MI. from his cell storage bank. The T-9 cell line originated from a high grade-anaplastic astrocytoma induced in Fischer rats by treatment with N-methyl-N-nitrosourea (MNU) (Schmidek *et al.*, 1971). Stock cultures were established in 25 cm2 tissue culture flasks and maintained in complete serum

supplemented medium (DMEM and RPMI 1640/Hams F12) replacement every 3rd day and the passing of cells every 6th day at a 1:100 split ratio.

Culture Conditions

Cells were cultured in a Hotpack CO_2 Incubator which was maintained at 5% CO_2, 37°C and constant humidity. Both neonatal astrocytes and astrocytoma cells were split into multi-well (6 well) culture plates (GIBCO Labs) containing 3 ml of complete medium. This medium helped to assure cell attachment. The complete medium, serum supplemented, was changed the following day and replaced with chemically defined medium (CDM). CDM HL1A was composed of DMEM supplemented with 1% (v/v) HL-1 supplement, 400μM glutamine, gentamycin at 10 μg/ml, glucose at 1 mg/ml, $CaCl_2$ at 175 μg/ml and $MgSO_4$ at 125 μg/ ml. CDM HL1B was also composed of DMEM and contained the same supplementation as HL1A but also contained hydrocortisone at 1.6 μg/ml, prostaglandin F2-alpha at 440 *n*g/ml, putrescine at 78 μg/ml, basic-fibroblastic growth factor at 8.8 *n*g/ml and myelin basic protein at 440 *n*g/ml. This HL1B medium was a slight modification of that originally proposed by Morrison and deVellis in 1981 as a CDM that initiates differentiation of astrocytes. HL-1 supplement contains 29 μg/ml total protein with 15 μg/ml insulin and contains no additional growth factors or glutamate.

GLU Uptake Experiments

Neonatal astrocytes and astrocytoma cells used in these studies were seeded at 6-12,000 cells/ml into 3 ml of complete, serum-supplemented, media at initial plating. The medium was changed the following day to serum-free CDM. Three days later the CDM was replaced with fresh CDM of

the same type and NGF added. NGF was added at 500 *n*g/ml or serum added at 10% (300 μl/3 ml medium). Cells were incubated in NGF or serum for 48 hrs prior to the addition of GLU. Each GLU concentration that was studied (2.5 and 25μmol) contained 3 μCi of [^{3}H]-GLU per 3 ml of culture medium per petri dish. [^{3}H]-GLU had a specific activity of (1 μCi/ 40*p*mol). GLU specific activity was established at 1μCi/2.5-25μmol. The standard incubation period for GLU uptake was set at 10 min at room temperature (RT) (20-25^{o}C). During the uptake incubation, cell culture plates were placed on a Lab-Line rotary orbit shaker (60 rpm) to assure dispersion of GLU. The incubation was stopped by removing the [^{3}H]-GLU containing medium and rinsing the cultures twice in a HEPES buffered saline solution, pH 7.4, which contained the same concentration of unlabeled GLU. Each rinse was for a period of 10 min and was also carried out on a rotary shaker. Following this rinse procedure the cells were extracted by placing 2 ml of a freshly prepared solution of 1M perchloric acid into each well. Cells were extracted for 10 min, again utilizing the rotary shaker. Cells were then given a post-extraction rinse using 2 ml of 0.1M Tris buffer, pH 7.4. This rinse solution was combined with the extract solution. Throughout these procedures cells remained attached to the culture plate. GLU uptake data were recorded as counts per minute (cpm) of extract and then normalized using the specific activity of GLU and cpm/protein concentration of extract and reported as *n*mol GLU/mg protein/time (10 min). The experiment was designed to compare uptake of GLU by neonatal astrocytes and astrocytoma cells cultured in two different chemically defined media, HL1A and HL1B, and to determine the effects of NGF, serum and hypothermia (Table 1). Three or more replicate cultures were preformed for each study.

Scintillation Analysis

The procedure was carried out using a Packard 300 scintillation counter. Uptake extract solution (0.8 ml) was added to Aquasol II scintillation fluid (10 ml).

Protein Concentration

The Bio-Rad method of protein analysis was used. Extract uptake solution (0.8 ml) was combined with 200 μl of reagent and absorbance checked at 595 nm. This was compared to a standard curve using Bovine IgG and quantitated accordingly. The average protein concentration for T-9 cultures was 36.8 μg/ml. and 38.8 μg/ml for NR-1 cultures.

Statistical Analysis

Determination of statistically significant differences between experimental groups was performed utilizing the Students-t test. Differences were considered to be significant when p values of ≤ 0.05 were obtained. Standard error of adjusted means (SEM) was also determined and reported where significance was indicated.

Results

Effects of Medium on net GLU Uptake

The net uptake of GLU (2.5 and 25μmol) by NR-1 and T-9 cells in HL1A and HL1B CDM is compared. Cells were cultured in both media and incubated with GLU for 10 min. There was a significantly lower uptake of GLU (2.5 and 25μmol) by NR-1 cells as compared to T-9 cells cultured in both HL1A and HL1B CDM (Table 1). In addition, there was a significant difference in GLU uptake (2.5 and 25μmol) between HL1A and HL1B media by both cell types.

Effects of NGF on GLU Uptake

The effect of NGF (500 *n*g/ml) on GLU uptake by NR-1 and T-9 cells cultured in HL1A and HL1B CDM following a 10 min incubation is illustrated in Table 1. NGF (500 *n*g/ml) was not found to significantly change the net uptake of GLU (2.5 and 25μmol) by either NR-1 nor T-9 cells.

Effects of Fetal Calf Serum on GLU Uptake

Table 1 illustrates the effects of serum (100 μl/ml medium) on GLU uptake by NR-1 and T-9 cells cultured in HL1A CDM following a 10 min incubation. The addition of fetal calf serum (FCS) in place of NGF was found to significantly increase the net uptake of GLU (25μmol) by NR-1 cells. However, serum had no significant effect on GLU uptake by T-9 cells.

Effects of Hypothermia on GLU Uptake.

The study of GLU uptake by NR-1 and T-9 cells at temperatures <4°C was performed to determine the presence or absence of an active uptake system. Cell cultures were placed on ice for 90 min prior to and during a 10 min incubation with GLU (25μmol). Hypothermia was found to cause a significant decrease in the uptake of GLU (25μmol) by T-9 cells cultured in HL1A CDM while significantly increasing the GLU uptake by NR-1 cells under the same conditions. This study found that under these culture conditions two different cell types can be shown to have different GLU uptake systems. The results indicate that GLU uptake by T-9 astrocytoma cells is an active uptake process.

TABLE 1. *GLU uptake by neonatal astrocytes and anaplastic astrocytoma cells cultured under varying conditions and treatments.*

Culture Conditions and Treatment (concentration, temp, medium, treatment)	GLU Uptake by Cell Type	
	NR-1	**T-9**
	*n*mol GLU/mg protein/10 min)	
25μmol GLU, RT, HL1A	86.0 (2.4)	1726 (307)
2.5μmol GLU, RT, HL1A	8.3 (0.9)	234 (30)
25μmol GLU, RT, HL1B	55.0 (1.3)	1384 (47)
2.5μmol GLU, RT, HL1B	6.3 (0.9)	156 (14)
25μmol GLU, RT, HL1A, NGF	76.7 (12.3)	1581 (38)
2.5μmol GLU, RT, HL1A, NGF	6.3 (0.9)	179 (2.6)
25μmol GLU, RT, HL1B, NGF	63.0 (7.6)	1476 (77)
2.5μmol GLU, RT, HL1B, NGF	5.0 (0.6)	175 (31)
25μmol GLU, RT, HL1A, Serum	217.0 (13.6)	2218 (127)
25μmol GLU, 4°C, HL1A	153.0 (15.7)	153 (6.1)

Statistically significant differences in GLU uptake are shown between NR-1 and T-9 cells cultured in either HL1A or HL1B medium, between T-9 cells cultured in HL1A and HL1B medium as well as NR-1 cells cultured in different media, between serum treated NR-1 cells and untreated cells cultured in HL1A medium and in T-9 cells in response to cold temperature. All comparisons were made using the Students-t test with $p<0.05$. Standard error of the mean (SEM) is given in brackets.

Discussion

The purpose of this study was to compare GLU uptake by NR-1 astrocytes and T-9 astrocytoma cells under varying conditions and treatments, with a special emphasis on the effect of NGF on GLU uptake. This was generated by NGF's reported ability to initiate morphologic differentiation (Greene and Tischler, 1976; Marushige *et al.*, 1989) as well as its reported ability to arrest proliferation (Marushige *et al.*, 1987) of different cell types. These reports generated a hypothesis that NGF may be beneficial in the treatment of tumors of the nervous system. Because of the importance of how NGF might affect the uptake of GLU by tumor cells differently than it does normal cells, this study draws a comparative picture of the differences in cell type.

Medium preparation is a very important factor in any study where morphology may be an intricate part of the expected outcome. Morphology has been previously reported to affect amino acid neurotransmitter uptake (Wilkin *et al.*, 1983). Different medium preparations have also been shown to initiate morphologic differentiation (Morrison and De Vellis, 1981). These reports added to our interests of how different chemically defined media, which influence morphologic differentiation, might affect GLU uptake.

There have been excellent studies on glutamate uptake by astrocytes and astrocytoma cells previously reported in the literature, and some have indicated that glutamate uptake was greater in astrocytes derived from brain areas in which there was greater glutaminergic input(Drejer *et al.*, 1982, 1983; Nicklas and Browning, 1983; Hanson, 1986; Waniewski and Martin, 1986;

Schousboe *et al.*, 1987; Flott and Seifert, 1991). However, fewer studies have been reported showing differences in GLU uptake between astrocytes and astrocytoma cells studied under identical *in vitro* conditions. One previous study has shown that the glutamate carrier in astrocytic primary cultures exhibits a substrate specificity which is somewhat different from that of glioma cells (Balcar *et al.*, 1987).

In this experimental model there was a significantly lower uptake of GLU by neonatal astrocytes as compared to astrocytoma cells (Table 1). This suggests that one of the factors present in the CDM medium may be preferentially acting to decrease the uptake of GLU by neonatal astrocytes. Upon examining the individual constituents of the CDM used in these experiments, it appears that this action may be related to the presence of insulin in the medium.

The insulin receptor is a tetrameric glycoprotein consisting of two 95 kD β -subunits and two 135 kD α- subunits held together by a disulphide bond. The β-subunit of the insulin receptor induces tyrosine kinase activity (Kasuga *et al.*, 1982). This receptor-mediated kinase activity results in autophosphorylation as well as the phosphorylation of other appropriate substrates. Cultured brain cells have been shown to express this type of insulin receptor.

However, insulin's action on the uptake processes of the amino acid neurotransmitters may be more involved than just activation of receptor-mediated tyrosine kinase. Insulin has also been shown to mediate some of its biologic effects through other membrane-bound cyclic AMP-dependent and independent protein kinases (Marchmont and Housley, 1980; Walaas *et al.*, 1981; Heyworth *et al.*, 1983). Therefore, the sequence of insulin's action starts when insulin binding switches on receptor-mediated tyrosine kinase; this

activated receptor then becomes autophosphorylated. This action, in turn, increases the activity of a kinase system to phosphorylate other substrate target proteins, setting off a cascade of biological responses (Ebina *et al.*, 1985; White *et al.*, 1985; Riedel *et al.*, 1986). In addition to this increase in protein kinase activity, there are reports that insulin also inhibits [Ca^{++} Mg^{++}]-ATPase (McDonald *et al.*, 1982). Therefore, the cellular changes that are brought about as a result of insulin's interaction with the cell membrane include not simply its well known function in glucose transport but also its role in phosphorylation, dephosphorylation processes as well as in calcium mobilization.

Other consequences of the initial autophosphorylation of the insulin-receptor could be a cascade of cyclic AMP which could activate protein kinase A leading to phosphorylation of serine and threonine residues or a phosphoinositide cascade leading to increased levels of inositol trisphosphate (IP_3) with subsequent activation of protein kinase C following calcium mobilization. Therefore, the dramatic difference in GLU uptake between astrocytes and astrocytoma cells cultured in insulin-containing CDM may be due to the phosphorylation and subsequent inhibition of the GLU receptor-protein binding, brought about by insulin receptor-mediated activation of tyrosine kinase. In addition, the inability of the T-9 rat astrocytoma cells to respond to insulin, in a like manner, may be due to an insulin-dependent depression in the number of insulin receptors, brought about by a receptor down-regulation mechanism.

NGF treatment did not cause a significant change in the uptake of GLU by either NR-1 neonatal astrocytes nor T-9 anaplastic astrocytoma cells (Table 1). However, NGF showed, except for one case, a consistent increase in GLU uptake by cells cultured in HL1B (Table 1). This could be explained by a

synergistic effect being produced by NGF and basic-FGF, a constituent of HL1B but not HL1A, which changes the affinity of the GLU receptor such that uptake becomes concentration-dependent. NGF and basic-FGF may both act through a receptor-linked kinase-mediated phosphorylation. But the phosphorylation site would be expected to differ from any phosphorylation mediated by insulin receptor-linked tyrosine kinase which might cause a decrease in the affinity of the GLU receptor in NR-1 astrocytes. NGF and basic-FGF could mediate phosphorylation at this second site and cause the GLU receptor-protein's tertiary structure to change there by affecting the receptor's affinity for GLU binding. This phosphorylation produced by the combined action of NGF and basic-FGF, dependent on 25μmol or higher GLU concentrations, could be responsible for the increase of GLU uptake by NR-1 cells seen in this study. NGF did not cause a significant change nor a consistent increase in the uptake of GLU by NR-1 astrocytes at 2.5μmol GLU concentration cultured in HL1B nor by astrocytes cultured in HL1A medium. NGF also had this type of effect on GLU uptake by T-9 astrocytoma cells. However, none of these changes noted were significant.

These speculations on the action of NGF and basic-FGF are supported by the data showing a significant increase in the uptake of GLU by NR-1 astrocytes in the presence of serum (Table 1). Fetal calf serum is known to contain growth factors whose receptors are associated with tyrosine kinase activity. The availability of high energy phosphate could explain activation of tyrosine kinase processes and could help explain an increased uptake of GLU following incubation with serum for 48 hrs.

The hypothermia study was performed in order to block active uptake . However, under conditions of hypothermia (temp <4^{o}C) NR-1 neonatal astrocytes showed a significant increase, while T-9 anaplastic astrocytoma cells

showed a significant reduction in GLU uptake (Table 1). These data support the fact that GLU uptake by T-9 astrocytoma cells is an active uptake process dramatically different from that expressed by NR-1 neonatal astrocytes. GLU uptake by NR-1 astrocytes appears to be actively suppressed during culture in these two CDM at room temperature,which is speculated to be caused by insulin in the media, or NR-1 astrocytes might express an as yet unidentified receptor, active at cold temperatures and unique to astrocytes.

In summary, GLU uptake by NR-1 astrocytes is markedly lower as compared to GLU uptake by T-9 astrocytoma cells. This response is suspected to be caused by insulin-dependent mechanism, which may inhibit the uptake of glutamate by neonatal astrocytes through an active phosphorylation of the high affinity glutamate receptor. NGF caused no significant change in GLU uptake by NR-1 neonatal astrocytes or T-9 anaplastic astrocytoma cells. GLU uptake by T-9 astrocytoma cells is an active uptake process that is not significantly affected by NGF or serum.

The significance of this study is that *in vitro* glutamate uptake by normal astrocytes is quite different than that of astrocytoma cells cultured under the conditions presented here. NGF does not significantly affect the net uptake of GLU by astrocytoma cells. However, NGF and basic-FGF may act synergistically to increase GLU uptake by neonatal astrocytes and astrocytoma cells. This information should be helpful in understanding functional differences between normal astrocytes and astrocytoma cells as well as in the development of appropriate therapeutic agents for the treatment of brain tumors as well as the prevention of seizures and the prevention of neuronal cell death.

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Chapter 7

Summary and Conclusions

Oncogene Expression and Cell Proliferation

Carcinogenesis is an extremely complicated pathologic process. In the brain, carcinogenesis is even more complicated because it can interfere with and disrupt normal neurophysiology. Seizures, as well as other neuropsychiatric symptoms could, therefore, be induced by brain tumors. Alkylnitrosourea induced brain tumors in the rat provide an excellent animal model for the study of brain tumors in humans and other animal species. This model provides researchers the opportunity to study the complete pathogenesis of brain tumors from initiation through progression. In addition, the combination of *in vivo* and *in vitro* studies, using this model, allows researchers to gain information on the molecular mechanisms responsible for cell transformation and tumor progression, as well as to study treatment and prevention.

It was not long ago that the discovery of tumor-causing genes called oncogenes gave renewed hope that cancer might be prevented and controlled by regulating the expression of certain genes. Later a set of related genes, termed proto-oncogenes or cellular oncogenes (c-*onc*), were found to normally exist in the genome of higher animals. Since then, these cellular oncogenes have been found to code for two classes of protein. One class of proteins acts at the nuclear level, while the other acts in the cytoplasm and cytoplasmic membrane. However, the true function of many oncogenes remains unknown. The oncogene products have been assumed to act on

specific nuclear and cytoplasmic cell targets because of their distribution and localization within the cell. In brain tumors *src, ras, sis, neu* and *erb* oncogenes have been found to express protein products which are related to signal transduction. These proteins are present in the cytoplasm and membrane compartments of cells (Rohrschneider and Gentry, 1984). Oncogene products, which are components of the signal transduction pathways, in turn play an important role in controlling normal growth and differentiation. Other oncogenes have been found to express protein products with a nuclear distribution. These proteins are assumed to have a nuclear function (Eisenman *et al,* 1985). In brain tumors, the nuclear acting proteins of the *myc* and *fos* oncogenes have been identified. Expression of these oncogene proteins has been found to correlate to changes in cell growth (Campisi *et al,* 1984; Kelly *et al,* 1983;)

These reports just mentioned suggest that oncogenes may be involved in the initiation of carcinogenesis. Therefore, this information led quickly to the hypothesis that chemical agents which initiate cancer might act by mutating proto-oncogenes to produce activated oncogenes. These activated oncogenes in turn could be responsible for the abnormalities observed in growth control and differentiation. This hypothesis has been confirmed, in fact, in several types of tumors produced in rats and mice by chemical carcinogens. This hypothesis especially proved true for those agents which could cause base substitutions at specific sites in oncogenes. However, there remains a considerable variation in experimental tumor studies. Cell heterogeneity, between cells of tumors in the same tissues, different tissues or different species, all show variation as to the type and extent of mutation and oncogene involvement. In addition, there are other gene targets, which are also exposed to mutations and could play as important a role as the

oncogenes. These targets are represented by the tumor suppressor genes and transcriptional regulatory sequences (Weinstein, 1987). Therefore, it is difficult to assume that mutations of proto-oncogenes are the major mechanism of initiation. Given the number of signal transduction pathways and the overall complexity of growth regulation and differentiation, it has been suggested that more than 300 proto-oncogenes may exist in the mammalian genome (Weinstein, 1988). As a result, oncogenes and oncogene products may represent only a small fraction of a huge complex association of mechanisms responsible for normal cell function, differentiation and growth.

In addition to changes in oncogene expression, many tumors also exhibit a number of karyotypic variations, such as translocations, deletions and amplifications (Barbacid, 1986). These genetic abnormalities have been characterized in tumors for quite some time. The chromosomal changes appear to influence carcinogenesis through increased gene dosage. Tumors have long been reported to express a tremendous variety of aneuploidy and chromosomal variation. Therefore, it appears that what is common among tumor cells is definitely their heterogeneity.

One of the principal objectives of this study was to determine if nerve growth factor (NGF) had an effect on proliferation. The results presented in Chapter 2 indicated that NGF does affect the proliferation of astrocytoma cells. NGF has been shown by others to induce a rapid but transient increase in the expression of the *fos* oncogene product in PC12 cells (Kruijer *et al*, 1985). Transcripts of *fos* mRNA can be detected 5 min after treatment with NGF (50 *n*g/ml) and are maximally abundant at 30 min and then are found to decrease (Kruijer *et al*, 1985). Other agents and conditions have also been reported to induce the expression of the c-*fos* oncogene. These include cyclic-AMP and epidermal growth factor, as well as K^+ depolarization. However, artificial

glucocorticoids, which also induce differentiation, do not induce c-*fos* oncogene expression (Kruijer *et al*, 1985). NGF induces PC12 cells to acquire properties of sympathetic neurons (Greene and Tischler, 1976; Dichter *et al*, 1977). In contrast to NGF, artificial glucocorticoids induce PC12 cells to acquire the characteristics of chromaffin cells (Schubert *et al*, 1980). Therefore, future differentiation studies involving the effects of NGF on astrocytoma cells should examine the expression of the c-*fos* oncogene to determine if NGF concentration (5 μg/ml and 50 *n*g/ml) makes a difference in the extent of c-*fos* oncogene expression. In addition, morphologic differentiation by NGF at high levels and low levels of NGF concentration should be examined to determine changes and differences in the induction process.

Astrocytoma cell proliferation was investigated by using acridine orange (AO) flow cytometry. The technique used had the advantage of characterizing cells in the cell cycle, while at the same time distinguishing distinct cell populations by their expression of genes including oncogenes in the cellular genome. Future studies should determine how these changes in gene expression, especially changes in c-*fos* and c-*myc*, relate to changes in cell cycle and proliferation potential.

Non-dividing, quiescent cell populations (G0) are characterized by having a constant amount of DNA (2n) throughout their life span. Proliferating cell populations are characterized by changes in their DNA cycling and division into daughter cells. The content of DNA in proliferating populations of cells increases during interphase from the diploid chromosome complement (2n) to a tetraploid chromosome amount (4n). DNA synthesis normally occurs only during the S phase of the cell cycle. This S phase is preceded and followed by two separate stages of interphase which are characterized by their differences in amount of chromosomal DNA.

These separate stages of interphase can be distinguished as subpopulations and are referred to as G_1 and G_2, respectively. G_2 cells have twice the chromosomal DNA of G_1 cells and are quite easily identified. However, G_1 and G_0 cells have the same relative amount of chromosomal DNA and, therefore, can not be distinguished. Quiescent (G_0) cells in a proliferating population of cells are actually dead cells. Cells which make up a true quiescent (Q) subpopulation are, for the most part, G_0 cells. These cells are derived from G_1 cells but have withdrawn from the proliferating pool. However, G_0 can not be distinguished from G_1 by quanitation of DNA and, therefore, quiescent cell subpopulations are identified as G_0, G_1. However, quiescent cells differ in gene expression from cycling cells and therefore may be distinguished by characterization of ss and dsDNA. The G_2 phase (subpopulation of interphase) of the cell cycle is followed by the mitotic (M) phase and represents cell division. This M phase also contains cells with a 4n DNA content. Therefore, cells of the G_2 and M subpopulation also can not be distinguished by DNA content. As a result of DNA characterization by content and type (ssDNA, dsDNA), three distinct subpopulations of cells should be distinguished. Proliferating cell populations are composed of a G_0, G_1 subpopulation followed by a G_2 + M subpopulation. S represents cells in DNA synthesis and is found in the area between the two subpopulations. A quiescent population of cells, which are non-dividing, are also represented by G_0, G_1. However, this quiescent population is not followed by a G_2 + M subpopulation. In addition, cells in a quiescent population are characterized by their greater stage of differentiation and degree of gene expression. Therefore, quiescent cells should contain greater amounts of single-stranded DNA.

Flow cytometry is a relatively new development in biophysics which results in an efficient and accurate method for analyzing DNA in individual cells (VanDilla *et al*, 1969,1975). Flow cytometry has the advantage of measuring both single stranded and double stranded DNA (ssDNA, dsDNA) by quantifying the intensity of fluorescence emitted by DNA-bound acridine orange (AO) dye. AO bound DNA, excited with blue light, fluoresces red when bound to ssDNA and fluoresces green when bound to dsDNA. A population of cells can, therefore, be characterized by their ssDNA to dsDNA content. Each specific cell type is different because of a variation in gene expression. This variation can be characterized by variation in the amount of ssDNA by different regions of the genome and by the extent of gene expression . This variation in ss and dsDNA, which in turn relates to gene expression and differentiation can be detected by flow cytometry, while carrying out cell cycle analysis based on DNA content. A proliferating cell population, which is cycling, can be easily distinguished from a non-cycling, quiescent cell population. In addition, closely related cell types, which originate from a common precursor but which differ in proliferation potential and gene expression, can also be distinguished from one another. . Cell populations with greater proliferation potential and less differentiation will have a greater ratio of dsDNA to ssDNA than a cell population with less proliferation potential and greater differentiation. Cells characterized by using these types of data analysis are illustrated by a scatter graph histogram. These histograms clearly show the proliferation potential and degree of differentiation between cell populations.

The technique of flow cytometry shown here works very well to distinguish subpopulations of astrocytes and astrocytoma cells. Flow cytometry could be utilized to an even greater extent in future studies by use

of a dual laser system. This would allow for the detection of specific proteins, especially c-*onc* products, at the same time as cell cycle analysis.

The brain tumor model used here as an example, provides an excellent model for continued research into cell cycle kinetics and the molecular mechanism of action of reverse transforming agents like NGF.

Morphology and Intermediate Filaments

Alkylnitrosoureas, including MNU, impart their carcinogenic behavior by producing alkylation of DNA bases . MNU exerts its effects by generating a reactive intermediate released during its decomposition. The ultimate electrophile produced by this process is the methyldiazonium ion (Kleihues and Magee, 1973). The main product of MNU exposure has proven to be N7- and O^6-methylguanine. The N7-methyl alkylation of guanine is easily removed from chromosomal DNA by non-enzymatic depurination while the O^6-methyl alkylation is not (Kleihues *et al*, 1982). The potentially mutagenic product of MNU exposure has proven to be O^6-methylguanine (Singer, 1975). This DNA lesion leads to misincorporation of the uridine and adenine bases during RNA co-polymer synthesis (Gerchman and Ludlum, 1973). Although RNA polymerase normally makes a good copy of the DNA polynucleotide template, based on the Watson-Crick base-pairing model, the presence of O^6-methylguanine leads to extensive misincorporation. These nucleic acid base pair substitutions are believed to cause the mutagenic and carcinogenic events of this transformation. Such molecular events in the DNA may also, therefore, be responsible for the aberrant synthesis of IFs.

The promoter sequence and transcriptional startpoint of the GFAP IF gene (mouse) has been mapped out along with characterization of promoter

function (Miura *et al*, 1990). Three *trans*-acting binding sites of the promoter region have been defined by DNase I footprinting and identified as GF I, GF II and GF III. The GF III binding site has been found to be cyclic-AMP responsive. Cyclic-AMP functions in this instance as an enhancer binding protein for this specific promoter region. NGF is known to increase cellular levels of cyclic-AMP (Schubert and Whitlock, 1977; Cremins *et al*, 1986). Therefore, NGF may enhance the promotion of GFAP IFs through its generation of cyclic-AMP.

Mutations, specifically to the GF II binding site, have been found to drastically reduce promoter activity, while base substitutions in GF I and GF III were found to abolish the cell-specific expression of GFAP altogether (Miura *et al*, 1990). Loss of cell-specific expression has since been shown to often result in GFAP promoter expression even in non-GFAP producing cells (Miura *et al.*, 1990). The GFAP gene sequence has been analyzed and found to be heavily concentrated with guanine bases. The GF II promoter region, for example, is a 20 base pair sequence which contains a very high concentration (50%) of guanine. Mutations to this region could severely affect the expression of GFAP IFs. Therefore, exposure to MNU and the generation of O^6-methylguanine could not only result in changes of the protein structure of IFs, but could drastically reduce GFAP IF expression altogether. These mutational changes could also be responsible for more subtle changes in protein solubility characteristics as well as for changes in the expression of GFAP epitopes. Changes in epitope specificity have been reported to be the result of abnormal glycosylation (Hughes and Sharon, 1978). However, epitope expression and concentration may be adversely affected by additional mutations involving guanine.

Since their discovery, IFs have been reported to be constituents of the cytoskeleton and to play a structural role in cells. However, more recent reports have indicated that this may not be so. The microinjection of antibodies directed specifically against the IF protein has been reported to cause a collapse of the IF network without affecting the cytoskeleton and cell morphology (Klymkowsky *et al*, 1983). As a result of this, many alternative functions for IFs have been suggested. These functions assumed that IF subunit proteins are responsible for targeted cellular functions and that the formation of filaments only represented a storage and transportation form of a protein messenger that could be released from the filamentous polypeptide in response to intra- and extra-cellular signals (Traub, 1985).

Studies to determine IFs function have shown GFAP and vimentin to both be nucleic acid binding proteins (Vorgias *et al*, 1983). In addition, the N-terminal arginine residues of the IF protein subunits have been shown to play an important part in filament assembly and disassembly. These subunit proteins have been further shown to be processed for biological activity by cleavage of this arginine-rich region by a Ca^{++}-activated, neutral thiol protease, which also inhibits them from re-assembling into filaments (Traub and Vorgias, 1983,1984).

More detailed studies have reported an interaction of IFs with ribosomes. IF protein subunits have been shown to bind with high affinity to ribosomal RNA (rRNA). There is a preferential binding to 18s rRNA, which suggests that these IF protein subunits may be single stranded (ss) nucleic acid-binding proteins. The binding of these IF subunit proteins to rRNA was also found to be influenced by nucleic acid base composition. The subunit proteins were found to bind to DNA and RNA sequences with increasing intensity as a function of their guanine content (Traub *et al*, 1983).

The results of GFAP and vimentin immunofluorescence studies, presented in this report, illustrate and support these previous studies. The binding of vimentin and GFAP IF proteins to rRNA may be illustrated by the greater fluorescent intensity of the perinuclear and nucleolar regions of both neonatal astrocytes and anaplastic astrocytoma cells.

Cells showing nucleolar immunocytochemical-staining have morphologic characteristics suggesting them to be protoplasmic type astrocytes. Astrocytes showing a stellate morphology are more characteristic of fibrous astrocytes. Fibrous astrocytes did not show intense fluorescence of nucleoli, however, they did show intense cellular immunofluorescence as well as nuclear fluorescence. These results suggest that one IF protein may share GFAP and vimentin epitopes in common and be expressed by protoplasmic type astrocytes and anaplastic astrocytoma cells. In addition, the GFAP epitope of this IF protein may be preserved by paraformaldehyde fixation and degraded by alcohol fixation. This is further speculated to involve aberrant changes in glycosylation, which imparts epitope specificity.

The expression of IF proteins by MNU-induced astrocytoma cells may be further complicated by the transformation properties and molecular involvement of guanine and guanine base substitution. Fibrous type astrocytes may demonstrate an end-stage developmental process and therefore, may express IF proteins with GFAP-specific epitopes which differ from GFAP epitopes expressed by protoplasmic type astrocytes. This expression of GFAP would again be independent of its processing and glycosylation.

Besides the affinity of IF protein subunits for single-stranded rRNA and ssDNA, these protein subunits exhibit a strong tendency to react with histones (Traub, 1985). It has been reported that vimentin IF subunit proteins

induce a conformational change in histone-histone complexes rendering them more sensitive to enzyme-induced proteolysis (Traub, 1985). This finding also appears to be very significant and if truly valid, recognizes GFAP, vimentin and other IF proteins as messengers of nuclear regulation. Therefore, IF protein subunits may be capable of interacting with separate and different constituents of chromatin, DNA and rRNA nucleic acids as well as histone proteins. As a result, these interactions may involve two functionally different binding sites, a nucleic acid-binding site and an acceptor site for arginine rich polypeptides.

The significance of IF proteins having a nuclear function could be acknowledged by future studies showing their involvement in regulation of mitogenesis, gene expression or nuclear rRNA transport. Flow cytometry techniques could be a useful tool in substantiating these speculations.

Previous studies in the department of pathology and elsewhere have used high concentrations of NGF to induce morphologic differentiation in glioma cells (Marushige *at al.*, 1989). Normal concentrations of NGF in the rat brain have been reported to be 0.5-2.5 *n*g/g wet wt (Greene, 1977; Korsching *et al.*, 1985). In other studies involving NGF and epidermal growth factor (EGF) a much lower concentration has been reported to be used (Leutz and Schachner, 1981; Boonstra *et al.*, 1983). It appears that NGF at 5000 *n*g/ml may be acting on rat anaplastic astrocytoma cells to induce uniform differentiation characterized by the formation of intracellular bridges, activation of cytoskeletal proteins and reduction of the nuclear-cytoplasmic ratio as a result of an expanding cytoplasm with significant process development. However, this response may represent an overexerted action by NGF to induce a morphologically unique and uniform cell population but one that is still quite different than what would be expected of a heterogeneous population

represented by rat fibrous and protoplasmic astrocytic cell types. These cells are of neural ectoderm origin and, therefore, should be expected to express NGF receptors and respond. However, use of NGF as a reverse transforming agent should not unnecessarily include the morphologic differentiation of anaplastic astrocytoma cells to different appearing but otherwise abnormal cell types. Experimental studies involving NGF's action as a reverse transforming agent, therefore, might better be carried out at a more physiologic NGF concentration. The data presented here supports this recommendation and strongly suggests that NGF may act silently to facilitate the action of non-specific effectors on rat astrocytes and astrocytoma cells. Whether NGF acts in addition to other effectors of membrane transduction, acts by allowing certain specific biochemical reactions to take place, or by simply acting to organize that which would takes place irregardless of its presence is only for speculation at this stage of NGF research.

GLU and GABA Uptake Functions

Evidence has accumulated that indicates that the amino acid neurotransmitters (GLU and GABA) and glutamine flow between two metabolic compartments in the CNS. There is, in addition, a counter flow mechanism, whereby these neurotransmitters flow in one direction and are compensated by a flow of glutamine in the opposite direction. This concept of metabolic compartmentation represents and distinguishes metabolic differences between neurons and astrocytes (Waelsch *et al.*, 1964; Lajtha *et al.*, 1959; Berl *et al*, 1961). Since introduction of the compartmentation concept, it has been shown that the brain contains at least two different sub-compartments or pools, a large pool which contains glutamate and a smaller

pool which also contains glutamate (Garfinkle, 1972; Van Den Berg *et al.*, 1974; Clarke *et al.*, 1974). This smaller pool, however, contains a much smaller fraction of total glutamate and gives rise to the rapid formation of glutamine. However, glutamine from this pool does not, necessarily, contain all of the cell's glutamine (Cremer *et al.*, 1974).

There have been many reports indicating that numerous metabolites enter into each of these glutamate pools. Glucose and pyruvate, for example, have been found to enter both large and small pools where they can be metabolized to either glutamate or glutamine. On the other hand butyrate, citrate, bicarbonate, ammonia, succinate and others, as well as synaptic GLU and GABA, predominantly enter the small pool (Van Den Berg, 1972; Blazas *et al.*, 1972; Mohler *et al.*, 1974; Cheng and Bruenner, 1974; Clarke *et al.*, 1974).

GABA, transferred from one compartment to another, is compensated for by intermediates of the Krebs cycle. This metabolic interaction has been referred to as the GABA-glutamine shunt. In addition, the fact that GABA and glutamine function as links between two compartments has directed special attention to this shunt mechanism and its regulation. In mouse brain, this shunt has been shown to account for 8-10% of the total flux through the Krebs cycle. Glutamate has also been found to cycle. GLU is released by neurons and taken up by astrocytes. It is then metabolized to glutamine and cycled back to the neurons as glutamine, where it is hydrolyzed to regenerate GLU or GABA and ammonia. This cycling of GLU has often been referred to as the glutamate-glutamine shunt.

The importance of including a study on GLU and GABA uptake here is due to the possibility that neoplastic transformation interferes with this normal uptake process and that NGF treatment may normalize such an uptake process. Many would agree that differences in uptake would be

expected because these are in fact different cells. Molecular anatomy as well as physical and chemical characteristics of receptors and uptake processes are certainly as great a target for mutational events of alkylnitrosourea as intermediate filaments or components of the nucleus which regulate proliferation. There are key enzymes in this uptake process and metabolic system that are equally important. Glutaminase, glutamine synthetase and glutamate decarboxylase are key enzymes for GLU, glutamine and GABA respectively. These enzymes could constitute yet additional targets for mutational events that could be expected to drastically affect uptake.

Regional differences in uptake function of astrocytes have been well established by other studies (Monaghan *et al*, 1983). These differences in uptake are in turn brought about by differences in the extracellular environment and the influence of specific neurons. Therefore, studies on uptake function are best done in a controlled environment, where variation can be minimized. As a result, functional comparisons of GLU and GABA uptake undertaken in this study between astrocytes and astrocytoma cells should yield significant results pertaining to specific cell types and the effects of NGF on uptake processes. Additional information may also be gained in future studies relative to the causation of seizures and other types of impaired neurologic function which may accompany specific types of cellular induced pathology.

In the dog as well as the human the most prominent clinical sign at the time of initial presentation for astrocytomas is a seizure. However, seizures especially appear to be influenced by tumor location. Tumors occupying the temporal, parietal and occipital lobes of the brain have a high correlation to seizure induction. Tumors occurring in other areas of the brain have a high correlation to other specific behavioral symptoms. If GABA, the major

inhibitory amino acid neurotransmitter, was to decrease in synaptic concentration, due to an increase in astrocyte or astrocytoma cell uptake, it could be sufficient to induce seizures. GABA concentrations in the rat cerebral cortex are reported to be 0.5μmol/g wet wt. and in the human 0.8-2.3μmol/g wet wt.. In human glioma tissues GABA concentrations have been further reported to decrease to 0.5μmol/g wet wt.. This suggests that a significant change in GABA uptake and metabolism may exist.

GLU is the major stimulatory amino acid neurotransmitter. If synaptic concentrations were to increase due to a decrease in astrocyte or astrocytoma cell uptake, it also might be sufficient to induce seizures and result in brain damage (Sloviter and Dempster, 1985). However, GLU appears to have an even greater influence on mental function. Abnormal increases in synaptic concentrations of GLU have also been correlated to spreading depression and abnormalities in memory formation (Bures *et al.*, 1974; VanHarreveld and Fifkova, 1974; Cherkin and VanHarreveld, 1978; Hertz, 1979). The later in turn may suggest that abnormalities in astrocyte function may be directly related to symptoms of Alzheimer's disease and loss of immediate memory capacity. GLU concentrations in the rat cerebral cortex are reported to be 2.1μmol/g wet wt. and in the human 7.8-12.5μmol/g wet wt. In human glioma tissues GLU concentration has been reported to be 4.2μmol/g wet wt.

Decreases in GLU and GABA concentrations may result in important changes to compartmentation and in the availability of metabolites from the glutamate pools. If the small glutamate pool, for the immediate conversion to glutamine, is disrupted, the supply of amino acid precursors to neurons is also depleted. This suggests that glucose-derived glutamate would become the major resource for GLU and GABA generation. This also suggests that the large glutamate pool would increase and could become a source of

intermediary energy metabolism for these tumor cells. If nothing more, these speculations suggest that further experimentation is necessary in the area of GLU and GABA uptake.

The uptake studies presented here determined the uptake of GLU and GABA at 2.5μmol and 25μmol concentrations of GLU or GABA. The lower concentration was used to establish a normal appearing, resting concentration and to observe the net effects of NGF on high affinity uptake mechanisms. The higher concentration was used to determine uptake function at a stimulatory or inhibitory concentration and to observe NGF's effects on low affinity uptake mechanisms. It has been well established that high GLU concentrations at 50-70μmol are toxic and lethal to neuronal cells. Therefore, these experiments were expected to generate the most useful preliminary information in the shortest amount of time and at minimal costs.

These studies differ markedly from previous studies reported in the literature. Differences include transmitter concentration, neonatal astrocyte isolation and uptake methodology. A proliferating population of astrocytes was generated from 4 day old rat pups. Previous studies had used 1 day old rat pups and many times mixed cultures. The astrocytes used in this study were at their 3rd passage, 75 days or longer in culture. They were characterized as astrocytes by their morphology and GFAP immunofluorescence. Over 95% of these cells were observed to be positive for the astrocyte-specific intermediate filament GFAP. In addition, approximately 95% of these cells exhibited epithelioid morphology characteristic of protoplasmic astrocytes. These astrocytes were also positive for GABA uptake, an additional defining characteristic for astrocytes. Other studies on GLU uptake by neonatal astrocytes have reported uptake at 10 min to range from 10-100*n*mol/mg protein. This study in comparison reports 8-86

*n*mol/mg protein for the GLU study and 15-483 *n*mol/mg protein for GABA. However, attempting to compare this study to previous studies for quantifying of GLU and GABA uptake is not consistent with the intent and purpose of this study. One would actually be trying to study and compare apples to oranges. Previous studies on uptake have taken cultured cells and perturbed them by initially culturing them in unrestricted medium and then rinsing them in a restricted buffer solution once, twice or three times and then measuring uptake in this buffer. This dissertation reports on an uptake procedure for unperturbed cells cultured in a restricted chemically defined medium; a system of analysis that, I personally feel, is more sensitive, efficient and accurate in determining uptake. These data, therefore, must be compared to other uptake studies with a great deal of caution. The best comparisons to be made are between neonatal astrocytes and anaplastic astrocytoma cells within this study.

Characteristics of GLU uptake in astrocytes are unique. Uptake is reported to be greater in astrocytes than neurons. It is not electrogenic and can be concentrated above extracellular concentrations. Astrocytes for the most part, are considered to be a sink for GLU. Characteristics for GABA uptake by astrocytes are also somewhat unique. Astrocytes express an active high affinity uptake system. The sodium gradient is the driving force, making GABA uptake electrogenic. GABA uptake by astrocytes has its limits. It has been reported that GABA can not be concentrated above extracellular concentrations. However, GABA uptake in unperturbed neonatal astrocytes should be directed to the small glutamate pool for immediate conversion to glutamine. Personal consultation with other neurochemists has indicated that with a 10 min incubation metabolic influences should be negligible. The area of amino acid neurotransmitter uptake and metabolism definitely needs

further research. This area has tremendous potential for research which could result in a much better overall understanding of neuropsychiatric symptoms that accompany many neurologic diseases.

The studies presented here were planned and intended to compare neonatal astrocytes with astrocytoma cells and to determine the effects of NGF on the uptake of GLU and GABA. This information, it was hoped, could suggest whether or not NGF could influence amino acid neurotransmitter uptake and hopefully data could be shown to support its potential use as a reverse transforming agent for the treatment of astrocytomas. Fortunately or unfortunately, the data leave more questions than answers. For example, the effects of temperature on GLU uptake is completely opposite of any other reports in the literature. These data are clear and crisp and significant, yet, preliminary and premature to make profound statements. Nevertheless, the results clearly suggest the discovery of another GLU receptor and mechanism; one that would be the first to be unique to astrocytes. This is an exciting discovery. However, there are so many new variables that were established with this new protocol, that it simply necessitates further studies and documentation in order to establish its reality.

Conclusions

This dissertation presents a new method for the isolation and long-term culture of neonatal astrocytes. It also presents a new method of cell cycle analysis of neonatal astrocytes and anaplastic astrocytoma cells using acridine orange for characterizing cells by ss and dsDNA. In addition it presents a new method to determine amino acid neurotransmitter uptake by culturing cells in a restricted chemically defined medium and measuring the uptake by unperturbed cells.

From the experimental data it may be concluded that NGF has an effect on proliferation and morphology of anaplastic astrocytoma cells. NGF was found to not affect the proliferation potential of neonatal astrocytes. However, NGF was found to facilitate the generation of a quiescent pool of non-cycling astrocytoma cells. This is the first time that data supporting changes in the cell cycle,from a proliferating population to a quiescent non-cycling population, have been illustrated and reported in tumor cell populations. This is a very significant finding.

NGF was found to facilitate the induction of morphologic differentiation in neonatal astrocytes and anaplastic astrocytoma cells by GLU. This facilitation by NGF was at a low concentration that did not induce differentiation by itself. In addition, GLU at the concentration used, following NGF treatment, did not induce morphologic differentiation without NGF. This suggests that NGF may facilitate the action of other differentiation promoter agents, which act by triggering membrane-mediated events that lead to membrane transduction, which in turn is followed by morphologic changes characteristic of differentiation.

NGF was also found to induce GFAP expression in neonatal astrocytes and anaplastic astrocytoma cells. The GFAP epitopes induced by NGF in anaplastic astrocytoma cells were found to be fixation-sensitive. Paraformaldehyde fixation in cacodylate buffer preserved GFAP epitopes, while methanol fixation abolished GFAP epitopes in anaplastic astrocytoma cells.

In addition, NGF treatment did not have a deleterious effect on the uptake of the amino acid neurotransmitters GLU and GABA. NGF did not significantly affect GLU or GABA uptake by anaplastic astrocytoma cells as compared to neonatal astrocytes. However, NGF was found to significantly

decrease the uptake of GABA by neonatal astrocytes at 2.5 and 25μmol GABA concentrations. This may or may not be related to NGF's ability to stimulate protein synthesis or its facilitation of morphologic differentiation, which is speculated to be the result of cyclic AMP, phosphorylation and membrane transduction events.

The data accumulated in the studies on GLU uptake suggest that a 6th GLU receptor exists, which may be astrocyte specific. This receptor mechanism has been found to be actively suppressed under normal physiologic conditions and increased in response to temperatures <4°C. .

These studies have laid the ground work for future studies in normal and tumor cell biology. In addition, the new procedures presented in this dissertation should enhance our ability to study the neuropathologic and toxicologic effects of specific agents on astrocytes.

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