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IN VIVO AND IN VITRO EFFECTS OF DIFFERENTIATION FACTORS (NERVE GROWTH FACTOR AND GLIA MATURATION FACTOR ON NEURO-ONCOGENESIS

presented by

NARAYAN R. RAJU

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Narayan K. Raju

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ABSTRACT

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It was the purpose of this study to test the reverse transformation effects of NGF (nerve growth factor) and GMF (glia maturation factor) upon tumors of the NS (nervous system) induced by ENU (ethylnitrosourea). In the first experiment in vivo effect of NGF on transplacental ENU-induced peripheral nerve neurinomas in rats was investigated in a 12-month study. Of the 34 rats transplacentally exposed to ENU and followed by subcutaneous administration of NGF on days 12-16, 90-94, 210-214 post-partum, 16 (47%) rats were affected with peripheral nerve neurinomas as compared to 29 (85%) in the NGF-untreated group of 34 rats. The neurinoma reduction following NGF treatment was significant at $p < 0.01$. Seven neurinomas in the NGF-untreated group were shown to contain NGFR (NGF receptor) sites when tested with 192-IgG NGFR antibody, whereas none of the neurinomas in the treated group tested positive for receptor protein. The results indicate a positive correlation between the suppressive NGF effect upon neurinoma development and the presence of NGFR sites.

In a second experiment the in vivo effects of NGF and GMF on the anaplastic glioma T9 cell line were explored by transmission and scanning electron microscopy. After exposure to GMF, cells became slender with long branching processes that formed an interconnecting network. NGF induced flattened multipolar cells with web-like cytoplasmic processes that formed somatic links with adjacent cells via demonstrable junctional devices.

Internal cell features, common to both treatments, were characterized by formation of an elaborated cytoskeleton, intracellular organelles and junctional complexes. The nuclear cytoplasmic ratio was markedly reduced and the nuclei often contained single nucleoli. These changes are morphological indicators of differentiation.

In a third study ENU-induced NS tumors from the first experiment were characterized by immunohistochemistry. S-100 protein and GFAP (glial fibrillary acidic protein) were shown to be reliable markers for astrocytomas and astrocytic cells within mixed gliomas. Neurinomas and oligodendrogliomas were negative for GFAP. S-100 immunoreactivity in neurinomas was inversely correlated with the degree of tumor anaplasia.

The results of this study provide evidence that NGF and GMF (not tested in vivo) are potent reverse transformation agents for neuroepithelial tumor cells possessing proper membrane receptors.

DEDICATION

To my family

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NGF.....nerve growth factor

NGF-R.....nerve growth factor receptor

GMF.....glia maturation factor

ENU.....ethylnitrosourea

CNS.....central nervous system

PNS.....peripheral nervous system

CD.....cessarian derived

NACl.....sodium chloride

DAB.....diaminobenzidine

PC-12.....pheochromocytoma cell line

KD.....kilo dalton

KI-RAS-2...kirstein-ras gene

HA-RAS-1...harvey-ras gene

PAP.....peroxidase-anti-peroxidase

ABC.....avidin-biotin-complex

CHAPTER 1

A REVIEW OF NERVE GROWTH FACTOR AND ITS EFFECT ON NEURO-ONCOGENESIS

Nerve Growth Factor - Historical perspective

Nerve growth factor discovery was incidental to a bold and imaginative experimental manipulation with fragments of several neoplasms by Elmer Bueker (1948).[1] In his experiments, he implanted pieces of mouse sarcoma 180, Rous fowl sarcoma and mouse mammary adenocarcinoma into the body wall of 3-day-old chick embryos to study the effect of foreign grafts on the development of the nervous system. In these experiments, a fragment of wing bud was surgically removed from the implantation site. It had been previously shown that wing bud ablation resulted in a severe hypoplasia of ventral horn motor nerve cells in that spinal cord hemisection.[2] It became apparent that, following the ablation, hypoplasia of nerve centers resulted from death of differentiated neurons, and not from failure of precursor cell replacement. He postulated that there were unidentified physiochemical affinities which resided in the peripheral appendages (wing) that were responsible for inducing the outgrowth of nerve fibers from the spinal cord[3]. Thus, Bueker selected tumor cells of diverse genetic origin for the limb bud substitute, to assess the effect on the development and differentiation of spinal ganglion cells.

Within 3-5 days after tumor grafting, dorsal root ganglia sensory nerve fibers innervated the mouse sarcoma cells. Concurrently, the ganglia increased in volume when compared to the corresponding ganglia innervating the intact contralateral wing. Bueker repeated this experiment, placing the tumor fragments on the chorioallantoic membrane. In spite of physical separation from the underlying embryo, implanted tumor cells induced enlargement and profuse growth of the sympathetic nervous system.

He postulated that a soluble, diffusable substance in the tumor fragments had triggered the nerve fibers to sprout.

Cohen, Levi-Montalcini and Hamburger attempted (1954) to extract and purify from mouse sarcoma cells the fraction responsible for the nerve growth - promoting activity. This led to the unfolding of another important chapter in the discovery of nerve growth factor. Snake venom containing nucleic acid-degrading enzyme phosphodiesterase and other enzymes, was used to degrade the nerve growth-promoting fraction.[4] An unexpected result from the experiment revealed that snake venom itself contained a much higher fraction of the nerve growth-promoting molecule. This fact was later confirmed in 6 to 8 day old chick embryos.[5] A survey of organs homologous to venom producing tissues revealed that mouse submandibular salivary gland was the richest source of the compound which was later named nerve growth factor (NGF).[5]

Nerve Growth Factor. Nerve growth factor (NGF), a polypeptide extracted from salivary glands, exists in two different forms (7S and 2.5S NGF), depending upon purification procedures.[6] Both forms have identical physiological significance but differ slightly in molecular composition due to minor proteolytic damage during preparation.[7] The high molecular weight protein, (130,000, [or the 7S]) isolated by Varon et al[8] is a noncovalently linked complex of two alpha (2.7S), one beta-dimer (2.6S) and two gamma (2.5S) subunits, with the beta-dimer being the active molecule.[9] The weak association of the three subunits and their different isoelectric points allows separation by ion-exchange chromatography.[8] The complex subunits are structurally bound by one or two Zn^{2+} molecules. Removal of Zn molecules or pH < 5 or > 8 causes dissociation of the complex.[14,15]

The biologically active 2.5S NGF form is most commonly prepared from partially purified NGF according to the method of Bocchini and Angeletti.[9] The purified 2.5S NGF and intact beta-subunit differ only in an amino-terminal octapeptide and carboxyl-terminal arginine.[10,11]

The biological significance of the alpha and the gamma subunits is not known, but it has been postulated that they protect and store NGF in the salivary glands, presumably participating in the formation of the NGF from a larger biosynthetic precursor.[12,13]

To this day, the mouse submaxillary gland remains the richest source of NGF.[6] The NGF-type protein has also been purified to homogeneity from snake venom (MW 28,000, 2.2S), and both preparations, although differing minimally in molecular weights and sedimentation coefficient, possess similar biological properties and activities.[16,17]

The biological role of this salivary protein remains undefined, as well as its potential for access to the nervous system. Thoenen et al[18] and Green et al[19] ruled against the possible discharge of this NGF into the circulating blood and also demonstrated that a surgical removal of this organ had no adverse effect on the sympathetic and sensory cells. Increased production (10 fold) by male mice has no defined physiologic basis. Recent studies indicate that the synthesis of the protein molecule is controlled by testosterone and thyroxine, and a discharge of unusually high levels of NGF into the circulating blood of male mice during intraspecies fighting may be instrumental in triggering a more aggressive defensive or offensive behavior.[20,22] The mechanism of NGF discharge into the blood stream and the associated temperamental change in the male is not known.

Nerve growth factor is contained in varied amounts in many tissues of the body. Sympathetic ganglia, adrenal glands, kidneys, blood vessels, vas deferens and peripheral organs innervated by the sympathetic nervous system have higher levels of NGF than thymus, placenta, heart, spleen, liver and muscle.[23-26] The protein has been identified in fish, birds, reptiles, mammals, and amphibians.[23,27,28] Several cells in culture are capable of producing NGF, including 3T3 cells, SV40 cells, fibroblasts, myoblasts, melanoma cells, glioma cells, neuroblastoma cells and glioblastoma cells.[29-34]

NGF and Differentiation of Neurons: Nerve growth factor is required for the development and survival of sympathetic and sensory neurons in the peripheral nervous system and differentiation of neurons in the central nervous system.[6,7,35-37] Levi-Montalcini and Booker demonstrated the absolute NGF requirement of sympathetic neurons by a pharmacological experiment termed immunosympathectomy.[38,39] Daily injections of small amounts of anti-NGF antibody into neonatal rodents resulted in an almost complete disappearance of sympathetic chain ganglia.[40] Parenteral administration of NGF to neonatal animals produced an appreciable increase in size of the ganglia and earlier innervation of sympathetic end-organs by nerve fibers.[28] Levels of tyrosine hydroxylase and dopamine-hydroxylase, enzymes required for catecholamine biosynthesis, increase dramatically.[41]

During developmental innervation, the target tissue provides NGF, which is retrogradely transported from the synaptic junction to the neuronal soma, for maintenance of the differentiated state of the neurons.[42]

NGF bioassay: The two most common in vitro bioassays for NGF activity are the phenotypic changes elicited in rat pheochromocytoma cells (PC-12),[43] and the response of chick embryo ganglia to NGF exposure.[44] The chromaffin-derived neoplastic PC-12 clonal cells, not requiring NGF for survival, are provoked to sprout neurite-like processes by NGF exposure.[45] This phenotypic alteration is characteristic of differentiated neurons biochemically and ultrastructurally.[46] The second assay is based on the graded pattern of neurite outgrowth elicited from the chick embryo dorsal root ganglia after inoculation with NGF.[28,44] Although this assay is still in use, it has minor drawbacks in that the quantitation is somewhat subjective in scoring with limited accuracy and requires dissection of the embryonic chick tissues.[28] The PC-12 method, while being a simple and rapid test, offers more accurate results and is also highly sensitive and easily reproducible.[45]

Mechanism of action: The actual mechanism by which NGF exerts its multiple effects on target cells remains ill-defined. However, the initial step of the interaction between NGF and its target cells requires coupling of the protein to specific cell surface receptors[7,47,48] This complexation results in several rapid cell conformational and functional changes, with rapid uptake of metabolites, polymerization of tubulin to microtubules and stimulation of anabolic and catabolic pathways.[49] Several schemes have been postulated to explain the above NGF-mediated actions, including activation of secondary messengers such as cAMP and intracellular Ca^{2+} mobilization,[50] protein methylation,[51] phosphatidylinositol turn-over[52] and by induction of cyclic AMP-Ca^{+2}/phospholipid-dependent protein kinases.[53]

Within a few hours following coupling of NGF and receptor, the complex is internalized by endocytosis, partially engineered by microtubules.[48]

The endocytotic vesicles containing the hormone-receptor complex have two possible fates: (1) fusion with lysosomes leading to degradation or (2) translocation to nuclear receptors.[48,54] The latter pathway directly affects subsequent transcriptional events.[54] This concept is supported by detection of elevated levels of transcription-dependent enzymes, such as ornithine decarboxylase and tyrosine hydroxylase following administration of NGF.[12,41]

Nerve Growth Factor Receptor: The nerve growth factor receptor (NGFR) is a glycoprotein with a molecular weight of 70,000.[55,56] It is localized uniformly on cell bodies and growing neurites.[57] Two forms of NGFR have been identified; high affinity and low affinity NGFR with K_ds of 10^{-11}M and 10^{-9}M, respectively.[58] It is believed that the low affinity receptor may give rise to the high affinity receptor.[59]

NGF Gene: The first genetic information of NGF was achieved from the gene sequencing studies of mouse salivary NGF.[60] This discovery led to the identification of NGF cDNA and the cloning of the NGF gene in man and animals.[61,62] In humans, the NGF gene is located on the proximal short area of chromosome 1, and codes for 307 amino acid residues which, upon fractionation, yield the essential 118 amino acids that constitute the NGF protein.[63]

NGF and Neuro-oncology: Several investigations on the possible role of NGF and its antibody in the suppression of tumors arising from NGF responsive neural crest cells have been performed.[49,64-68] These experiments have shown that NGF has both maturation and differentiation

influence on neurectodermal tumor cells. Exposure of PC-12 pheochromocytoma cells to NGF resulted in cessation of mitosis, induction of neurite outgrowth,[45] induction of neuron-specific enolase and ornithine decarboxylase, and stimulation of amino acid uptake.[69-71] NGF also induced differentiation of human neuroblastoma line IMR-32,[66] and SH-SY5Y cells.[72]

In an in vivo experiment, NGF treatment of recipient rats bearing implanted undifferentiated F-98 glioma clone cells resulted in decreased tumor growth rate and increased life span of the animals.[73] Pretreatment of the malignant cells with NGF 24 hours prior to implantation showed a similar but less dramatic result.[73]

In a recently completed in vitro study in our laboratory, it was shown that treatment of anaplastic glioma T9 cells with NGF retarded the growth and induced morphological changes characteristic of a differentiated neuroepithelial cell.[74]

NGF and N-ethyl-N-nitrosourea-induced peripheral nerve neurinomas: It has been shown that NGF is capable of suppressing neurinomas in rats and mice transplacentally induced with N-ethyl-N-nitrosourea (ENU).[67,68,75,76] ENU selectively induces tumors of the nervous system in rats when exposed transplacentally as fetuses during late gestation.[77] A single dose of 50 mg/kg ENU administered via the lateral tail vein to pregnant rats at 20 days of gestation results in the production of neurogenic tumors in nearly 100% of the offspring.[77] Neurinomas of the trigeminal nerves appear as early as 20 days post-partum at which time the

lesions are classified as early neoplastic proliferations (ENP). By 90 days post-exposure, nearly 100% of the rats are affected.[67,76-78]

Administration of NGF either postnatally after optimal dose ENU exposure or transplacentally prior to optimal dose ENU exposure led to a significant reduction of ENP and trigeminal neurinomas at 90 days of age in the offspring.[67,76]

Ethylnitrosourea: Neurocarcinogenesis

Ethylnitrosourea (ENU) is a potent resorptive neurocarcinogen in a variety of rat strains.[79,80] It is a direct-acting carcinogen belonging to the Acyl-Alkyl nitrosomides which non-enzymatically activate to an ultimate carcinogen by dissociating rapidly in a slightly alkaline environment to release the reactive electrophil.[81] Transplacental administration of ENU to pregnant rats in late gestation or to newborns soon after birth selectively results in tumors of the central and peripheral nervous systems.[79,80,83]

The degree of sensitivity of the developing nervous system to ENU is directly related to the gestational age of the fetuses, the dose and route of ENU administration.[79,84] Susceptibility extends from the 12th day of gestation until 2 weeks after birth, with a maximum sensitivity at 15 days of gestation at which time predominantly neural tumors of the central nervous system will develop.[79,85] Fetal rats exposed to ENU after the 15 day gestation develop more peripheral nerve tumors.[71,76]

A teratogenic effect is observed when ENU is administered before the 12th day of gestation.[83,85] The degree of oncogenic effect declines postnatally, and after 30 days of age, the nervous system is relatively resistant to neoplastic transformation by a single dose of ENU.[83,86]

After an optimal transplacental ENU dose, tumors of the peripheral nervous system have the shortest latent period followed by those of the central nervous system.[83,85] The peak of sensitivity varies with different structures in the nervous system and is further dictated by age, species and strain.[79,82] In the CNS oligodendrogliomas, ependymomas, or mixed gliomas are primarily located in the hippocampus, subependymal areas, lateral ventricles, cerebral cortical white matter, thoracolumbar and lumbosacral region of the spinal cord.[86] Neurinomas of the trigeminal nerve are first to appear followed by those of spinal cord nerve roots, lumbar and brachial plexuses, and sciatic nerve.

ENU and DNA Alkylation: The one most important property of N-nitroso compounds is the capability of alkylating DNA bases.[87] Although alkylation can occur at various sites on nitrogen and oxygen atoms in the DNA bases, it is the ethylation of the O^6-guanine which is one of the prime suspects contributing to oncogenesis.[87,88] Other adducts also formed are O^2-ethylcytosine, O^4- and O^2-ethylthymine.[88,89] Although the major products derived from ENU reactions with DNA are ethylphosphotriesters, their contribution to mutagenic activity is not known.[87,89] It is known that most alkylating agents are either carcinogenic, mutagenic or both.[88]

The precise molecular mechanism directing mutagenesis by alkylating agents is still obscure; however, experimental evidence supports the hypothesis that base-substitution mutations arise via the formation of alkyl-DNA adducts that could direct the misincorporation of nucleotides during DNA replication.[89] The alkylation of O^6 position of guanine could cause a mutation during DNA replication resulting from a miscoding with thymine instead of with the complementary cytosine base via the DNA polymerase enzyme.[90]

Repair of Alkylated DNA and Mutation: The persistence of alkylated DNA base(s) during cell replication is necessary for mutation to occur. After an exposure to ENU, brain, liver and kidney have comparable levels of O^6-alkylguanine in DNA.[90] However, within a few hours, liver and kidney efficiently remove the DNA adduct by a catalytic activity which correlates with the levels of the repair enzyme alkylguanine transferase in these organs.[87] In the repair of O^6-alkylguanine by the catalytic transferase enzyme, the alkyl group (methyl or ethyl) is transferred to a protein residue without any structural damage to the liberated DNA.[91] The rate of O^6-alkylguanine loss from the brain (target organ) is considerably less efficient and corresponds to the low level of the repair enzyme activity detected in the developing nervous system.[90] The failure to correct the DNA damage provides the high risk for the persistence of the pre-mutagenic lesion in rapidly replicating cells such as the glia.

It is apparent, therefore, that alkylation of DNA, with persistence of alkylated adducts and subsequent DNA replication and cell multiplication are necessary for early initiation of tumor growth by the N-nitroso compounds.

Role of Oncogenes in N-nitrosourea induced neurogenic tumors

Investigations have shown the association of transforming genes (oncogenes) in chemically induced neoplasms in rodents.[92-94] Harvey-ras gene (Ha-ras-1), in methylnitrosourea-induced mammary carcinomas in Buf/N female rats,[92] the Kirsten ras gene (Ki-ras-2) in renal mesenchymal tumor in rats,[93] and the N-ras gene in thymic lymphoma in mice[94] have been constantly found to be activated during tumor induction.

Recently, two studies have attempted to show the possible role of *neu* oncogene in neuro/glioblastoma cell lines derived from tumors induced by ENU in BDIX rats[95] and in ENU induced Schwannomas in F344 rats.[96] Activation of this transforming gene is believed to be consequential to a point mutation in the transmembrane domain of *neu* gene product.[97] Further investigation is required to determine the exact relationship between *neu* oncogene and N-nitroso compound induced neurogenic tumors in rodents.

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CHAPTER 2

THE EFFECT OF NERVE GROWTH FACTOR (NGF) ON TRANSPLACENTAL ETHYLNITROSOUREA (ENU)-INDUCED NEUROGENIC TUMORS IN SPRAGUE-DAWLEY RATS

SUMMARY

To determine the effect of nerve growth factor (NGF) on the spectrum, incidence and latency periods of neurogenic tumors, with particular attention to the neurinoma (Schwannoma) incidence, specific pathogen-free, date-mated Sprague-Dawley rats were injected with 50 mg/kg body weight of ethylnitrosourea (ENU) on the 20th day of gestation. Pups were weaned on the 28th day and caged in sex-matched pairs. Forty, 60 and 80 micrograms of NGF was subcutaneously inoculated to 34 offspring on days 12-16, 90-94 and 210-214 post-partum. Thirty-four control rats, not inoculated with NGF, were maintained under the same conditions as the experimental group. In the NGF-treated group, 11/34 rats were affected with trigeminal nerve neurinomas compared to 18/34 in the NGF-untreated controls ($p < 0.05$). In the peripheral nerves, there were 5 and 11 neurinomas, respectively, in each group. When the total numbers of neurinomas (trigeminal and peripheral nerves) between these groups were compared (16/34 versus 29/34), the significance of neurinoma reduction due to NGF treatment showed a p value of < 0.01.

The spectrum, incidence and latency period of central nervous system (CNS) tumors between NGF-treated (20/34) and NGF-untreated (25/34) groups did not vary significantly. A majority of the tumors consisted of differentiated gliomas. These results are consistent with the previous studies in which it was demonstrated that ENU selectively induced differentiated tumors in the CNS of rats, and that NGF did not have any influence on these differentiated neoplasms.

Five trigeminal and 2 peripheral nerve neurinomas in the NGF-untreated group were shown to contain nerve growth factor receptor (NGF-R) sites by the avidin and biotinylated horseradish peroxidase complex

method, whereas none of the neurinomas in the NGF-treated group tested positive for the receptor protein.

The results obtained from this experiment lend support to the hypothesis that NGF has the capability to reduce the oncogenic consequences of ENU exposure by the process of maturation and differentiation of the transformed cells, and that this activity is dependent upon the presence of NGF receptor binding sites.

INTRODUCTION

Nerve growth factor (NGF) is a polypeptide composed of alpha, beta, and gamma fractions with a molecular weight of 131,500[1,2]. The beta subunit, (β-NGF, a dimer of 13,000 molecular weight), is the active molecule which possesses the nerve growth promoting activity.[3-6] Recent evidence indicates that NGF also has a neurotropic effect on the cholinergic neurons in the brain.[7-9]

In embryologic development, any tissue which will be innervated produces nerve growth factor. After binding with the specific nerve growth factor receptor (NGF-R) on the nerve fibers, NGF is internalized and retrogradedly transported to the neuronal cells in the ganglia.[10,11] Several biochemical changes occur in the cell following the coupling of NGF with its receptor, some of which include an increase in cAMP levels and mobilization of intracellular Ca^{+2},[12] phospholipid methylation,[13] and phosphatidylinositol metabolism,[14,15] and induction of cyclic AMP- and Ca^{+2}/phospholipid-dependent protein kinases.[16]

The locations of NGF-R on the neurons have been identified by the immunoprecipitiation technique using monoclonal antibodies to the NGF-R protein.[17] This receptor has been identified in sympathetic and sensory neurons,[6] Schwann cells,[18] peripheral neuroglial cells,[19] pheochromocytoma PC-12 cells,[20] and melanoma cells.[21] (Refer to Chapter I for additional information on NGFR.)

A single dose of 50 mg/kg body weight ENU given to pregnant female rats via the lateral tail vein on the 20th day of gestation resulted in the formation of neurogenic tumors in nearly 100% of the offspring.[22,23] Sequential evaluation of these neoplasms indicated that neurinomas of the trigeminal nerves were evident as early as 20 days after

exposure, with nearly 100% involvement by 90 days.[24] The lesions progressed from early neoplastic proliferations (ENP) or micro-tumors to grossly detectable macro-tumors attaining a peak of development by 7 months. Gliomas of the central nervous system (CNS) and neurinomas of the peripheral nervous system (PNS, spinal nerve) appeared between 6 and 7 months post-exposure. At the time of termination of the one year study, there was an increasing trend in the number and frequency of these neoplasms.[23,24]

Several investigations demonstrated that NGF had both maturation and differentiation influences on neuroectodermal tumor cells.[25-29] Exposure of PC-12 pheochromocytoma cells to NGF causes a rapid stimulation of Na, K^+-pump mediated K^+ influx,[25] development of excitable membranes,[26] neurite outgrowth and cessation of mitosis,[27] all biochemical characteristics associated with differentiated sympathetic neurons.[30] A similar phenotypic alteration was also induced in 1MR-32 human neuroblastoma cell line after NGF treatment.[29] Rats implanted with anaplastic glioma cells and treated with NGF had a significant reduction of tumor growth rate ($p < 0.025$) and increased survival time ($p < 0.00005$).[31,32] Pretreatment of the malignant cells with NGF 24 hours prior to implantation yielded a similar, but less dramatic result.[32]

In a recent *in vitro* study,[33] using NGF and glia maturation factor (GMF), it was shown that both these factors induced characteristic changes of cell morphology and growth pattern in an anaplastic glioma cell line T9. Nerve growth factor retarded cell growth rate and induced a flattened cytoplasm with numerous protruding processes forming somatic links with adjacent cells. GMF did not affect the cell growth rate. The GMF

treatment transformed the plump T9 cells into slender cells with long cytoplasmic processes forming a characteristic interconnecting network. The effect of NGF was persistent following withdrawal of NGF from the medium, whereas the phenotypical alterations induced by GMF were reversible. Interestingly, concomitant treatment with NGF and GMF had the combined effect of both factors. These experiments supported the hypothesis that both growth factors function as regulators of cell differentiation.

In two 90-day termination studies,[34,35] treatment of pregnant rats with NGF, prior to ENU exposure or postnatally following gestational ENU administration, resulted in a significant reduction of ENPs in trigeminal nerves of the offspring. The present experiment was an extension of these 90-day studies and designed to explore the persistence of the NGF effect on neurogenic tumor development following transplacental ENU exposure by determining changes in the incidence, latency period and spectrum of neurogenic tumors. Special attention was to be given to the final effect of NGF upon trigeminal neurinoma development complementing the results obtained from the 90 day termination study.[34,35] Testing of the neurogenic tumors for the presence of NGF receptors would further determine whether prospective changes in tumor spectrum and/or incidence were the consequences of NGF administration and interaction with the tumor cells.

MATERIALS AND METHODS

Preparation of NGF. β-NGF was isolated from salivary glands of male Swiss Webster mice and purified by the procedure described by Bocchini and Angeletti.[36] Samples were tested for biological activity by the PC-12 method.[37]

Ethylnitrosourea: ENU[a] was dissolved in citrate phosphate saline buffer (3 mM sodium phosphate and 2 mM citric acid in 0.15 M NaCl, pH 4.2) at a concentration of 10 mg/ml, and injected within one hour after preparation.

ANIMAL EXPERIMENTS

Eight date-mated 20 day pregnant Sprague-Dawley (CD) rats[b] were given a single slow dose of 50 mg/kg body weight ENU via the lateral tail vein as previously described.[19] The nursing rats, along with the pups, were randomly divided into 2 groups.

Group A: 34 offspring (18 males and 16 females) were inoculated with 40, 60, and 80 ug of NGF (dissolved in sterile 0.15 M NaCl solution) divided in 5 subcutaneous doses on days 12-16, 90-94, and 210-214 as previously described.[31,35,38] This method of NGF administration was chosen to facilitate slow absorption and to ensure prolonged action of the hormone on the ENU-transformed cells. The incremental dose levels (20 ug) allowed for an increase in age and body weight.

Group B: 34 offspring (15 males and 19 females), which served as a positive control, were exposed to ENU but did not receive NGF. They were

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[a] Serva Fine Biochemical, Inc., Garden City Park, New York, 11040

[b] Charles River Laboratories, Portage, MI

maintained under the same conditions as the experimental group. The pups were weaned at 28 days of age and housed in sex-matched pairs. The rats were fed autoclaved Purina Lab Chow 5010C, with water ad libitum. All animals were observed twice daily and weighed weekly throughout the experimental period.

Termination of the experiment at one year of age precluded any significant natural occurrence of neurogenic tumors in CD rats.[39] A NGF control was, therefore, not included. NGF is, of course, not considered to be a carcinogen. Rats either died as a consequence of neoplasms or were euthanatized due to progressive neurological signs and weight loss. All animals were necropsied as soon as possible after death or euthanasia. All lesions, serial sections of the brain, trigeminal nerves, and six selected segments of the spinal cord were fixed in 10% buffered-formaldehyde. For immunocytochemistry, similar tissues, acquired from all animals, were frozen in liquid nitrogen and stored at -70°C.

Histopathology: Brain, spinal cord, trigeminal nerves, lung, liver, heart, stomach, intestines and kidneys from each rat and all gross lesions were routinely processed, embedded in paraffin, sectioned at 5 u and stained with hematoxylin and eosin (H&E). Special stains used included Masson's trichome, periodic acid-Schiff (PAS) and Giemsa stains.

Development of nerve growth factor receptor positive control: The NGF-R positive control was developed in 7-day transected sciatic nerve in male Sprague-Dawley rats as previously described with slight modifications.[18] The rats were anesthetized by IP injection of Equithesin.[c] A 3 mm

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[c] Prepared by Department of Pharmacology, Michigan State University

section of sciatic nerve was removed near the tendon of the obturator internus muscle, and the proximal stump folded beneath the tendon to prevent regeneration.

Seven days post-surgically, the rats were euthanatized and both ends of the transected sciatic nerve, intact sciatic nerve from the opposite leg, and the surrounding innervated muscles were frozen on dry ice and stored at -70°C.

Immunohistochemistry: Detection of NGF-R. Cryostat sections of ENU-induced neurinomas, transected sciatic nerves and innervated muscles were rapidly fixed in 4% paraformaldehyde prior to exposure to 192-IgG NGFR monoclonal antibody[d] (5 ug/ml). The antibody was suspended in a solution of 100 mM potassium phosphate, 160 mM NaCl/5% heat-inactivated horse serum/0.02% NaN_3, adjusted to pH 7.5. After binding for 30 minutes, sections were washed in PBS (20 mM potassium phosphate/150 mM NaCl, pH 7.5), and incubated for 30 minutes at room temperature with biotinylated horse anti-mouse IgG immunoglobulin. The sections were then washed, treated with 0.3% H_2O_2 in methanol to quench endogenous peroxidase activity, and, after a 10 minute wash, incubated for 30 minutes with a complex of avidin and biotinylated horseradish peroxidase.[e] The sections were washed gently several times, incubated in 0.05% 3, 3-diaminobenzidine-/0.01% H_2O_2 for 10 minutes. The sections were rinsed in distilled water (5 minutes), counter stained with Gill's hematoxylin (1-2 minutes), cleaned in distilled water, dehydrated in graded alcohols, and mounted.

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[d] A generous gift from Dr. Eugene M. Johnson, Jr., Department of Pharmacology, Washington University School of Medicine, St Louis, MO

[e] Vector Laboratories, Burlingame, CA

STATISTICAL ANALYSIS: A chi-square test of independence was used to determine if there was a significant difference in the incidence, spectrum and latency of neurogenic tumors between the NGF-treated rats (Group A) and the control rats (Group B).

RESULTS

Incidence of Trigeminal nerve neurinoma

In the NGF-untreated group (positive control) 15 of the 34 animals had grossly visible trigeminal neurinomas, and 3 had microscopic trigeminal ENPs (53%)(Table 1-1). Most trigeminal nerves were bilaterally involved with the gross lesions varying from edematous and unevenly swollen foci to friable, reddish-brown hemorrhagic tumors (Figures 1-1, 1-2, 1-3).

Histologically, the neurinomas varied in their degree of anaplasia. The anaplastic neurinomas consisted of hyperchromatic polyhedral cells with round to oval nuclei containing dense nuclear chromatin and pale cytoplasm. These cells were arranged in undulating sheets, whorls and dense clusters (Figure 1-4). Mitotic figures ranged from one to two per high power field. Scattered pockets of hemorrhage and necrosis were also present (Figure 1-5).

The ENPs were classified according to the criteria previously described.[19,20] These foci, typically located in the proximity of the junction between the peripheral nervous system and central nervous system, were characterized by disorganized arrays of hyperchromatic cells haphazardly arranged in irregular sheets (Figure 1-6).

In the NGF-treated group, 10 of the 34 rats had trigeminal nerve neurinomas and one had an ENP (32%)(Table 1-1). The difference in incidence between control versus treated group was significant at $p < 0.05$. These data are supportive of NGF effect by a significant reduction in the number of trigeminal nerve neurinomas ($p < 0.05$).

The incidence of peripheral nerve (spinal nerve root) neurinomas between groups did not differ significantly ($p < 0.1$)(Table 1-2). Five of the 34 treated and 11 of 34 control animals developed neoplasms.

Table 1-1: The effect of NGF on trigeminal nerve neurinomas and ENP occurring after ENU administration. Group A, NGF-treated; Group B, NGF-untreated.

Group	Number of rats	Number of rats with neurinomas	Number of rats with ENPs	Total Number of rats with neurinomas	Age (days) range	Age (days) average
A	34	10	1	11(32%)	160-347	243
B	34	15	3	18(53%)	182-316	233

* $p < 0.05$.

Table 1-2: The effect of NGF on peripheral nerve neurinomas occurring after ENU administration. Group A, NGF-treated; Group B, NGF-untreated.

Group	Number of rats	Number of rats with peripheral nerve neurinoma	Age (days) range	Age (days) average
A	34	5 (14.7%)*	162-351	254
B	34	11 (32.4%)*	196-365	247

* $p < 0.1$

Table 1-3: The effect of NGF on the total number of neurinomas occurring after ENU administration. Group A, NGF-treated; Group B, NGF-untreated.

Total Group	Total Number of rats	Number of rats with trigeminal nerve neurinomas	Number of rats with peripheral nerve neurinomas	Total neurinomas
A	34	11 (32%)	5 (15%)*	16 (47%)**
B	34	18 (53%)	11 (32%)*	29 (85%)**

* $p < 0.1$

** $p < 0.01$

Table 1-4: Incidence and spectrum of neural tumors in rats after ENU administration.

	NGF Treated	ENU Alone
Total number of rats	34	34
rats with CNS tumors	20	25
Type of Tumor		
Tumors of CNS		
Oligodendroglioma	16	20
Mixed Glioma	11	9
Astrocytoma	5	7
Meningioma	2	2
Glioependymoma	0	2
Total	34	40
Rats with PNS Tumors		
Neurinoma	16	29
Total number of tumors	50	69
Total number of tumor-bearing rats	32	32
Average number of tumor/rat	1.56	2.16

When the total neurinomas (trigeminal and peripheral) were compared between the groups, the effect of NGF on neurinoma development was highly significant ($x^2 = 3.72$, $p < 0.01$)(Table 1-3).

Incidence of CNS tumors

In the central nervous system, the proportion of rats developing tumors in both groups was similar; 20/34 in Group A versus 25/34 in Group B (Table 1-4)($p > 0.5$). However, due to multiplicity, the total number of neurogenic tumors in both groups exceeded the number of rats affected (Table 1-4). The number of tumors per animal ranged from 1 to 4 (an average of 1.56 in NGF-treated group and 2.16 in the ENU control). In the experimental group, 8 rats had both neurinomas and CNS tumors, whereas 14 rats had both types of tumors in the control group.

The average survival time of rats with the tumors is given in Table 1-4. The spectrum, incidence and latency period of CNS tumors did not differ significantly between the NGF-treated and untreated groups (Table 1-4). A majority of CNS tumors were differentiated gliomas. These results are consistent with previous studies which indicated that ENU selectively induces differentiated tumors in the CNS of rats,[22-24] and that NGF did not have any influence on these differentiated neoplasms.[31]

Classification of CNS Tumors

The tumors were categorized as described previously.[23,24] The gliomas in both groups were located in the hippocampus, periventricular areas, subcortical white matter, cerebral cortex, basal ganglia, and infrequently in cerebellum and medulla.

Oligodendrogliomas: These were the most common tumors in the CNS and were equally distributed between brain and spinal cord. Most spinal cord oligodendrogliomas were grossly detectable and preferentially located in the upper cervical, lower thorax, and lumbosacral areas. The lesions were pale, gelatinous to wet, reddish-brown, hemorrhagic foci (Figure 1-7). Histologically, the well-differentiated neoplastic cells were round, with scant cytoplasm and dense nuclei, and were sometimes arranged in small clusters with "honeycomb-like" patterns supported by thin fibrovascular stroma (Figure 1-8). The periphery of oligodendrogliomas blended into the adjacent brain neuropil.

Mixed Gliomas: Tumors in this category consisted of a mixture of differentiated astrocytes and oligodendrocytes (Figure 1-9), with either type predominating a single lesion. These were often located in the cerebral cortex, the hippocampus and, rarely, in the cerebellum.

Astrocytomas: The predilection site for astrocytomas corresponded to that of oligodendrogliomas. Most were microscopic in size and consisted of well-differentiated cells, rich in cytoplasm with oval to round nuclei (Figure 1-10). Mitoses and anaplasia were mostly inapparent.

Glioependymomas: Two of these neoplasms were identified in the ependymal region of the brain and contained proliferating glial cells and ependymal cells, forming vague rosettes.(Figure 1-11).

Meningiomas: Of 4 meningiomas, one appeared to involve the entire cerebral hemisphere containing areas of hemorrhage and necrosis (Figure 1-12). Histologically, the neoplastic cells invaded the cerebral cortex and had a moderate rate of mitosis. Isolated areas of necrosis were

observed. The remaining three meningiomas only compressed the cerebral hemispheres, but no invasion was recognized (Figure 1-13).

Optic Nerve Glioma: The observable lesion progressed from a slight epiphora and conjunctivitis in the right eye at the initial observation to an almost complete proptosis of the eyeball within 5 days (Figure 1-14). The tumor consisted of intraocular (neurinoma) as well as extraocular (glioma) cellular proliferation cells (Figure 1-15).

Non-neural neoplasms: Non-neural tumors included three mammary gland adenomas, one thymic lymphosarcoma, one thyroid follicular adenoma, three renal myxofibrosarcomas, one nephroblastoma, one renal adenocarcinoma, and two ameloblastic odontomas. The latter is a very rare tumor in animals, which has not been previously described in rats.

Nerve Growth Factor-Receptor: The hypothesis that suppression of neurinoma development by NGF is dependent upon the presence of NGFR was investigated. Forty-five neurinomas from both groups (29 from untreated and 16 from treated) were tested for NGFR by the immunoperoxidase method. Five trigeminal and 2 peripheral nerve neurinomas (7/29) in the untreated group were positive, while all neurinomas in the treated group were negative. Staining variability ranged from focal positive areas to almost complete staining of the complete sections (Figure 1-16). Elapsed time post-sectioning had no effect on the specificity or consistency of the procedure. There was no nonspecific background staining. The NGF-R positive control (transected sciatic nerve) and NGF-R negative neurinomas are illustrated in Figures 1-17 and 1-18.

Figure 1-1 **Ventral view of rat brain. Notice bilaterally swollen trigeminal nerves (arrows).**

Figure 1-2 **Dorsal view of rat trigeminal nerve affected with anaplastic neurinoma (1); brain reflected dorsally (2).**

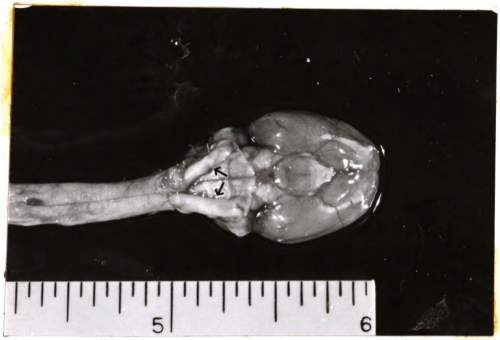

Figure 1-1

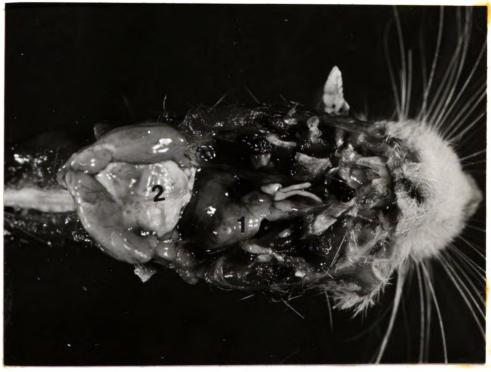

Figure 1-2

Figure 1-3 **Ventral view of rat brain showing bilateral anaplastic trigeminal nerve neurinoma (1); hemorrhage (2).**

Figure 1-4 **Photomicrograph of an anaplastic neurinoma illustrating hyperchromatic polyhedral cells and mitoses (arrow). H & E. X 400.**

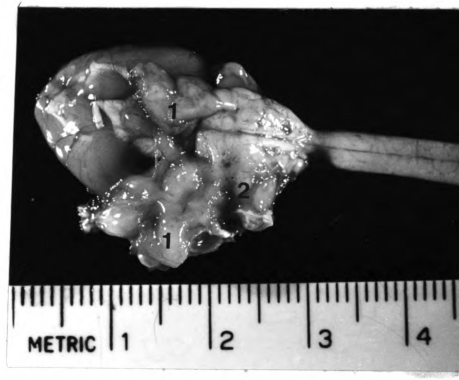

Figure 1-3

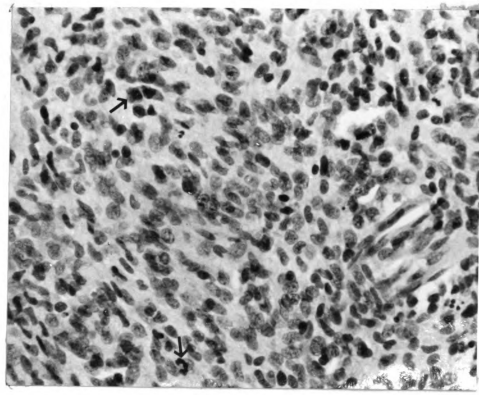

Figure 1-4

Figure 1-5 **Photomicrograph of an anaplastic neurinoma showing areas of necrosis and hemorrhage. H & E. X 400.**

Figure 1-6 **Photomicrograph of an early neoplastic proliferation (ENP) in a trigeminal nerve. Arrow indicates CNS-PNS junction. H & E. X 160.**

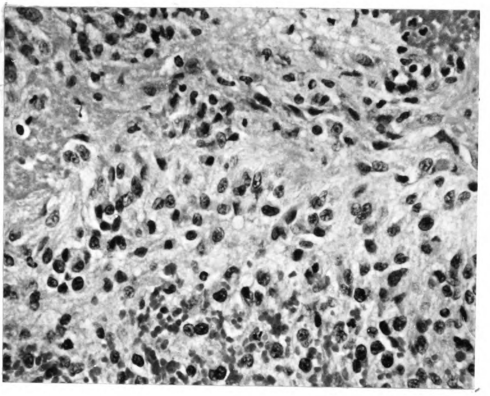

Figure 1-5

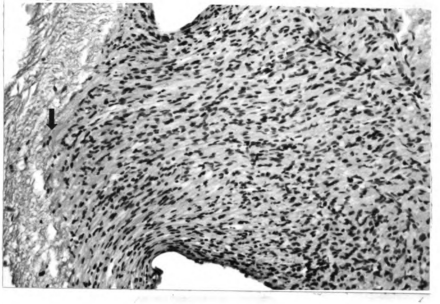

Figure 1-6

Figure 1-7 **Gross appearance of an oligodendroglioma in the lumbo-sacral region of a spinal cord.**

Figure 1-8 **Photomicrograph of an oligodendroglioma. Notice small round cells with scant cytoplasm and dense nuclei arranged in a "honeycomb"-like pattern. H & E. X 160.**

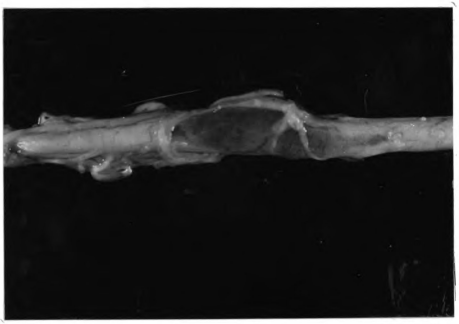

Figure 1-7

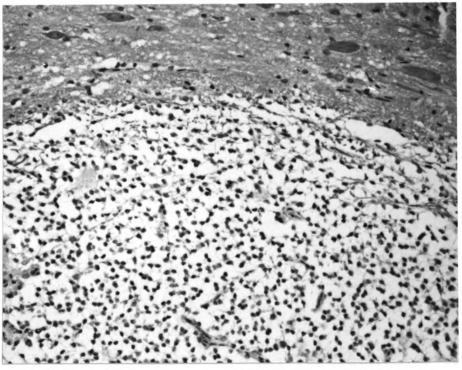

Figure 1-8

Figure 1-9 **Photomicrograph of a cerebral mixed glioma illustrating mixed population of neoplastic cells. H & E. X 400.**

Figure 1-10 **Photomicrograph of cerebral astrocytoma. H & E. 400 X**

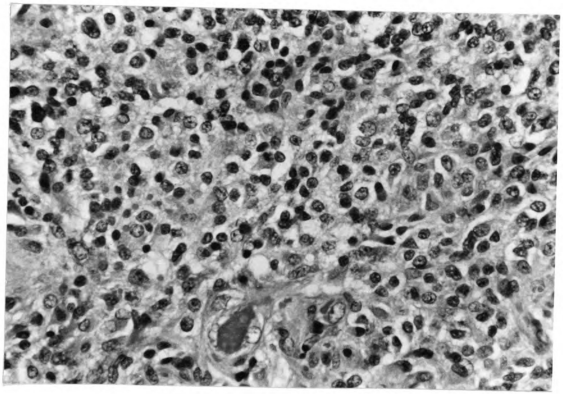

Figure 1-9

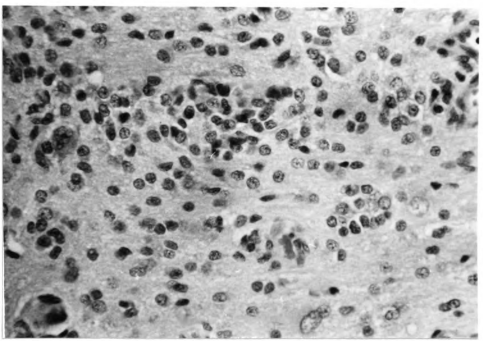

Figure 1-10

Figure 1-11 **Photomicrograph of a cerebellar glioependymoma. Neoplastic cells are arranged in short chains and rosettes. H & E. X 400.**

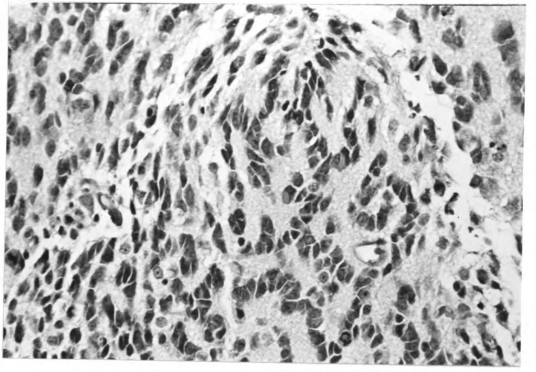

Figure 1-11

Figure 1-12 **Gross appearance of a meningioma involving the entire dorsal cerebral hemisphere.**

Figure 1-13 **Photomicrograph of the meningioma in Figure 1-12. Notice oval to fusiform neoplastic cells forming nebulous whorls. H & E. 400 X.**

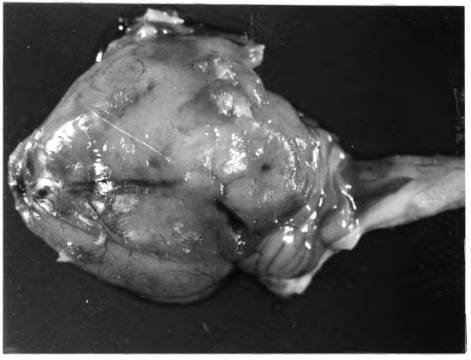

Figure 1-12

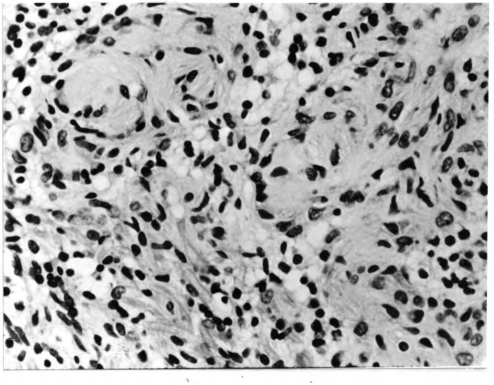

Figure 1-13

Figure 1-14 **Ventral view of a rat brain showing bilateral trigeminal nerve neurinoma (1); optic nerve glioma (2); globe (3).**

Figure 1-15 **Photomicrograph of optic nerve tumor. Glioma (1); intraocular neurinoma (2); retina (arrow). H & E. X 60.**

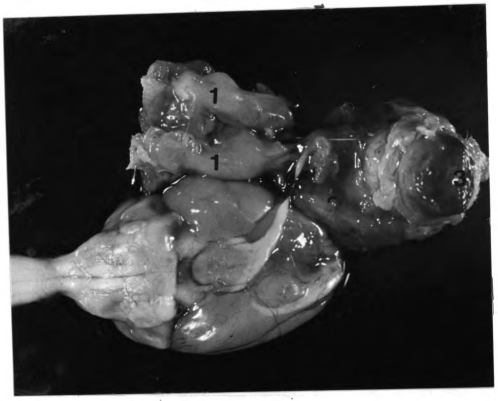

Figure 1-14

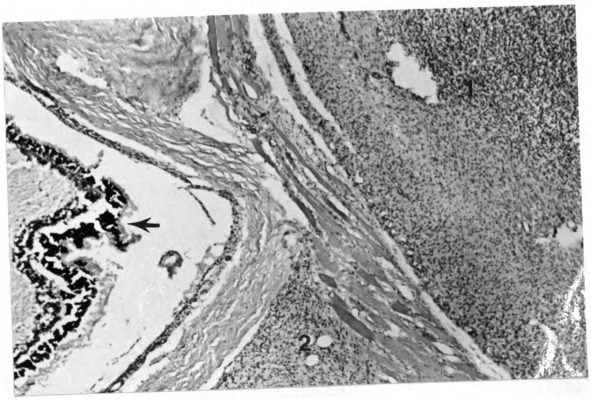

Figure 1-15

Figure 1-16 **Photomicrograph of a neurinoma immunostained for nerve growth factor receptor (NGFR). Notice strong NGFR positive cytoplasmic reactions in neurinoma cells. ABC method. Hematoxylin counter stain. X 100.**

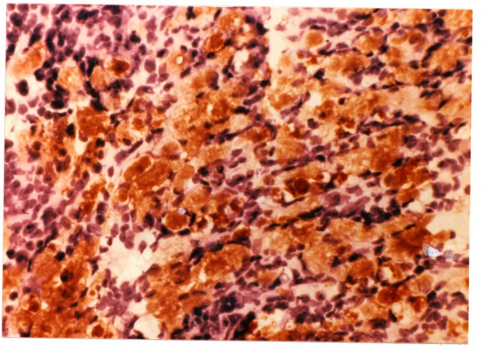

Figure 1-16

Figure 1-17 **Photomicrograph of 7-day transected rat sciatic nerve immunostained for NGFR (positive control). Schwann cells have strong positive reaction around the cytoplasmic rim. An unstained blood vessel (center). ABC method. X 100.**

Figure 1-18 **Photomicrograph of a neurinoma immunostained for NGFR. Notice all neoplastic cells are negative. ABC method. Hematoxylin counterstain. X 100.**

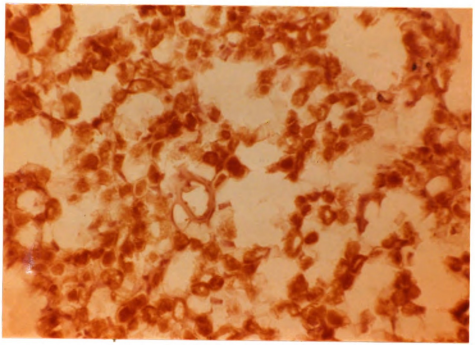

Figure 1-17

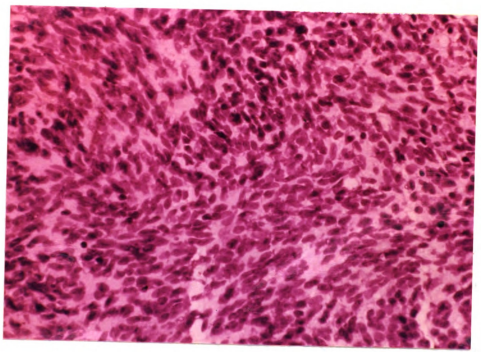

Figure 1-18

DISCUSSION

This study supports the hypothesis that NGF reduces neurinoma development in rats transplacentally exposed to ENU and complements the previous 90 day studies.[34,35] It establishes the persistence of the neurinoma-reducing effect previously recognized in the 90-day studies.

Since the mechanism of NGF interaction with neuronal cells is dependent upon the presence of NGF-Rs,[10,11] the ability of the NGF to induce differentiation of the anaplastic neuroepithelial cells is also dependent upon binding of the hormone to the receptor molecules. In the present study, NGF-R protein was present only in the NGF-untreated group and none of the 16 neurinomas in the NGF-treated group were positive. Since there was an association between NGF-R and the administration of NGF, and since the number of tumors in the NGF-treated group was inversely related to the presence of NGF-R, we suggest that tumors with NGF-R binding sites may respond to the exogenous NGF administration and this treatment results in suppression or differentiation of anaplastic cells.

Immature Schwann cells of peripheral nerves are the target cells for ENU transformation.[23] Studies by Vinores and Koestner,[34,38] and Camp *et al*,[35] documenting the reduction of ENPs by NGF, given prior to ENU exposure, might be explained by enhanced maturation of immature Schwann cells, thereby reducing the target cell population. NGF given after ENU exposure suggests that it is also capable of suppressing the phenotypic expression of the transformed cells.

One mechanism important in the process of transformation is formation and persistance of premutagenic O^6-ethylguanine adducts in DNA.[40] The developing nervous system is prone to retain this DNA lesion, since there is a virtual lack of the O^6-ethylguanine repair enzyme.[40]

Selective gene suppression via the epigenetic pathway might explain the protective effect of NGF on ENU-initiated cells. The epigenetic pathway is activated by two possible mechanisms: (1) by means of secondary messengers following the coupling of NGF to the cytoplasmic receptors, (2) internalization and binding of NGF to the nuclear receptor triggering the induction of transcriptional-dependent enzymes via mRNA. In support of this mechanism, NGF induces specific transcription dependent enzymes, including tyrosine hydroxylase,[41] dopamine-beta-hydroxylase,[42] and ornithine decarboxylase[43] in normal and neoplastic neural cells after NGF exposure.

Although there was also a slightly lower incidence of gliomas in the NGF-treated rats (20 versus 25), the difference was not significant statistically; however, a trend cannot be eliminated. A significant effect upon these differentiated gliomas by NGF was not expected since previous studies determined a selective NGF effect solely for anaplastic glioma cells.[31,32]

In the present experiment, it was not determined whether a single NGF treatment or a combination of all three treatment schedules were responsible for the suppressive effect on neurinoma incidence. Further studies are needed to evaluate the effectiveness of the individual NGF exposure times on neurinoma development.

The results of our study provide a promising new regime in the management of tumors derived from neural crest cells. In theory, therefore, beneficial effects from NGF therapy might be derived when used in pharmacological doses in conjunction with the conventional therapeutic approaches including surgery, radiotherapy and chemotherapy.

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CHAPTER 3

THE *IN VITRO* EFFECTS OF NERVE GROWTH FACTOR (NGF) AND GLIA MATURATION FACTOR (GMF) ON ANAPLASTIC GLIOMA T9 CELL LINE: SCANNING AND TRANSMISSION ELECTRON MICROSCOPY STUDIES

Summary: The objective of this study was to explore the ultrastructural components of the anaplastic glioma T9 cells following exposure to glia maturation factor (GMF) and nerve growth factor (NGF). While the internal cytoskeletal elements, as examined by transmission electron microscopy (TEM), were essentially identical in both treatments, scanning electron microscopy (SEM) revealed distinctly different cell surface characteristics. GMF exposed cells were characterized by three dimensionally cylindrical and multipolar cell bodies with several long, slender cytoplasmic processes which appeared to form an interconnecting network with neighboring cells. These processes were sparsely decorated with thin, hair-like structures. NGF induced short and broad cell bodies with wide cytoplasmic "foot" processes. Fine, web-like configurations, or lamellipodia, sprouted from the ends of these processes which were ramified by filamentous, hair-like filopodia and secondary branches.

Internal architecture, common to both treatments, included elaborate arrays of 5 nm microfilaments, 10 nm intermediate filaments and 25 nm microtubules dispersed throughout the perikaryon. An abundant golgi apparatus, SER, RER, fat globules, free ribosomes and an extensive network of tortuous mitochondria were evident in treated versus control cells. Junctional complexes (zonula occludens) between the adjacent cells were a prominent feature with both treatments.

When compared to control cells, the nuclear:cytoplasm ratio was markedly reduced in treated cells. The nuclear contour appeared smooth with occasional irregular indentations. Often, there was a single nucleolus with condensed chromatin material.

Introduction

Glia maturation factor (GMF) is an acidic protein which is capable of promoting growth and differentiation of glioblasts.[1] It is endogenous to the brain and has recently been purified to homogeneity.[2] Studies have shown that GMF can induce proliferation and morphologic differentiation of Schwann cells[3] and astrocytes[1] in cell cultures.

Nerve growth factor (NGF) is a basic protein which is necessary for the development and maintenance of the sympathetic neurons and the development of sensory neurons in the peripheral nervous system and for differentiation of the neurons in the central nervous system.[4-7] (A detailed review of NGF is contained in Chapter 1 and 2 of the dissertation.)

In vivo and *in vitro* studies have shown that NGF and GMF have both suppressing and differentiating effects on neuroectodermal tumor cells.[8-14] After NGF exposure, PC-12 pheochromocytoma cells cease mitosis, sprout neuritic processes, increase the rate of metabolite uptake, increase activity of the Na^+, K^+ pump and induce transcription-dependent enzymes normally associated with terminally differentiated neurons.[8,9] A similar conformational change was also observed when neuroblastoma cell lines were treated with NGF.[10,15].

Recent studies have shown that GMF is capable of reversing or suppressing neoplastic characteristics of Schwannoma and glioma cells.[3,14] We demonstrated changes[16] in rat T9 cells which developed distinctively different characteristics in growth pattern and cell morphology after treatment with NGF and GMF. This chapter describes the scanning (SEM) and transmission electron microscopic (TEM) evaluation of the changes precipitated by NGF and GMF treatments.

Materials and Methods

Preparation of NGF and GMF. Beta-NGF was isolated and purified from male mouse submaxillary glands by the procedure of Bocchini and Angeletti,[17] and the biological activity assayed by the PC-12 method.[18] Partially purified GMF was prepared from bovine brains as described by Lim and Miller.[19]

Cell culture. Stock monolayer cultures of rat T9 glioma cells[20,21] were maintained in DMEM[a] containing 10% fetal bovine serum[b], 4 mM glutamine, 100 units/ml of penicillin and 50 ug/ml of streptomycin in a humidified chamber with 7% Co_2 at 37°C. A single cell suspension was prepared by trypsinization, and cells ($1\text{-}3x10^4$/dish) were seeded in 5 ml of the stock culture medium onto Lux culture dishes[c], (Permanox, 60 mm-diameter). The dishes were coated with 50 ug/ml of polyD-Lysine (M_r 30,000-70,000) for 5 minutes,[22] washed with Hank's balanced salt solution and used immediately. One day after seeding, the culture medium was replaced with 5 ml of chemically defined serum-free medium, HL-1[d], containing NGF (5 ug/ml) or GMF (5 ug/ml). The control cultures were maintained in the identical medium without NGF or GMF. HL-1 contains DMEM:F12 base, HEPES buffer, insulin, transferrin, testosterone, sodium selenite, ethanolamine, saturated and unsaturated fatty acids and stabilizing proteins. It was supplemented with 4 mM glutamine.

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[a] Dulbecco's modified Eagle medium. Gibco Laboratories, Grand Island, New York.

[b] Armour Pharmaceutical, Kankakee, Illinois.

[c] Miles Scientific, Naperville, Illinois.

[d] Ventrex Laboratories, Portland, Maine.

T9 cells treated with GMF, in particular, and with NGF tended to partially detach from the culture surface and round up with slight mechanical shocks. It became necessary to develop a special procedure for medium exchange. The cells treated with GMF were fixed one day earlier (at 3 days) in order to keep the cell structures intact during cell culture and to prevent mechanical damage during the process of washing and fixation. After two days of culture in the experimental medium with daily medium change, 2 ml of the medium was replaced by 2 ml of the fresh medium containing either 25 ug of NGF or 25 ug of GMF. On the following day, 1 ml of fresh medium containing 25 ug of NGF was added without withdrawing the existing medium to the culture of cells with NGF, and 1 ml of the medium without the factor was added to the control culture. The control cells which were cultured in the experimental medium for 3 days and 4 days were identical in the morphological characteristics.

Transmission Electron Microscopy (TEM)

After 4 days of culture, the control and the growth factor treated cells were processed for TEM by the following procedure:

1. The media was gently extracted and immediately replaced by 5 ml of 0.1M PO_4 for washing.
2. After two careful washings, the cells were fixed in 4% glutaraldehyde in 2% (0.1M) sucrose PO_4 (5 ml of 8% glutaraldehyde + 5 ml 0.2M PO_4 with 4% sucrose) for 15 minutes at room temperature.
3. The fixative was washed three times with 0.1M PO_4 + 2% sucrose.
4. This was followed by osmification of the cells with 1% OSO_4 for 30 minutes.
5. After one wash with 0.1M PO_4, the cells were rinsed once with deionized distilled water.
6. 1% uranyl acetate was then added to the culture dish for 30 minutes.
7. The cells were dehydrated in graded ethyl alcohol (30, 50, 65, 75, and 95%) for 5 minutes at each change followed by 3 treatments with absolute ethanol.
8. The dehydrated cells were rinsed twice for 2 minutes in propylene oxide.
9. Finally, the cells were embedded in 1 part propylene oxide + 3 parts resin mixture overnight inside a hood and then polymerized in an oven for 2 days.

The area of epon-embedded cells to be examined by TEM was first observed and outlined under a phase contrast microscope. Several 1-2 mm

sections were cut by wire-saw and glued to the tip of resin stubs suitable for mounting on the ultra microtomes for sectioning. Thin sections were stained with uranyl acetate and lead citrate, and examined with a Philips 300 transmission electron microscope.

Scanning Electron Microscopy (SEM)

Cells were plated on poly-D-lysine coated Lux Thermanox tissue culture coverslips[c] and incubated in Falcon 24-well plates with 0.5 ml/well (2 cm^2) of the culture medium. The slightest disturbance during media change tended to float these coverslips in the solution. Although careful direct dropwise addition of the medium to the coverslips minimized the flotation, it did not prevent the dislodging of the cells from the coverslips. The procedure was modified to overcome this problem: after 2 days of culture in the experimental medium, 0.2 ml of the medium was replaced by 0.2 ml of the fresh medium containing either 2.5 ug of NGF or 7.5 ug of GMF; on the following day, 0.1 ml of the fresh medium containing the same amount of NGF or GMF was added without withdrawing the existing medium. After 4 days of culture, the cells were fixed by carefully adding 0.5 ml of 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) on top of the medium. The cells were postfixed with 1% osmium tetroxide, dehydrated with successively increasing concentrations of ethanol, and critical-point dried and sputter-coated with a 300-A^o layer of gold-palladium. Cells were observed with a JEOL JSM-35C scanning electron microscope at 15 Kv.

Results

Scanning Electron Microscopy

Control T9 cells: The cells were large and polyhedral in shape with a tendency to form clusters. Generally, these cells exhibited a haphazard overlapping pattern of growth which often obscured individual cell bodies (Figure 2-1). The cell edges were studded with few minute finger-like processes or microvilli. On higher magnification (Figure 2-2), the cells consisted of relatively broad cell bodies with broad bands of "foot"-processes. Variably-sized microvilli were randomly scattered along cytoplasmic borders as well as over the cell bodies. Cells in the process of division was not an uncommon observation (Figure 2-3).

GMF-treated Cells

Cells exposed to GMF showed remarkable phenotypic alterations characterized by greatly reduced size of cell bodies and development of several long, slender cytoplasmic processes which appeared to form an interconnecting (or communicating) network with neighboring cells (Figure 2-4). These processes were decorated with sparse, thin, hair-like structures.

NGF-treated Cells

NGF treatment, on the other hand, revealed more dramatic changes in the T9 cells. In addition to suppression of cell division, there were several characteristic cytoplasmic configurations. The cell bodies were short and broad with relatively broad cytoplasmic ("foot") processes (Figures 2-5, 2-6). These processes, similar to those in GMF-exposed cells, formed somatic links with the processes from the adjacent cells. Whenever a cell was individualized, these processes appeared to

serve as an anchoring device (Figure 2-7). Fine, "paintbrush"-like configurations or lamellipodia sprouted from the terminal end of these "foot" processes. These lamellipodia, in turn, were ramified by filamentous, hair-like filopodia and secondary branches, giving the entire structure a "crown-like" configuration (Figures 2-8, 2-9).

Transmission Electron Microscopy

T9 Control Cells. As depicted in Figure 2-10, T9 control cells were large, multipolar cells with large nuclei in proportion to the plump cytoplasm devoid of processes. There were scattered, small, round to oval mitochondria, attenuated smooth and rough endoplasmic reticulum (SER, RER), golgi complexes, abundant ribosomes and glycogen granules and perinuclear intermediate filaments (Figure 2-11). These cytoplasmic organelles appeared clustered close to the perinuclear region and were absent in the lamellae. The nuclear membrane appeared smooth with occasional areas of indentations. The nucleus contained multiple nucleoli with clumped chromatin.

GMF-treated cells: These cells had an elongated shape with multiple extensions (processes) and a decreased nuclear size (The nuclear:cytoplasmic ratio was morphometrically determined (Figure 2-12, 2-13). The nuclear membrane was smooth with irregular shallow clefts. The heterochromatin varied from coarsely condensed to irregularly tortuous with marginated nuclear chromatin. Dispersed among the elaborate cytoplasmic organelles were large, prominent spiralling mitochondria, stacks of distended SER, RER, golgi apparatus, myelin bodies, ribosomes and glycogen granules. The edges of the cells had abundant, variably-sized, slender, "finger"-like or filamentous processes. Cells lying adjacent to one another were opposed at the plasma membranes (Figure 2-12). Cells distant

to one another were in contact by interdigitation of the filamentous processes (Figure 2-14). Higher magnification of the perinuclear region revealed numerous linear arrays of 10 nm intermediate filaments (IFs), intermingled and intimately connected with the cytoplasmic organelles (Figure 2-15). The IFs were arranged parallel to the larger 25 nm microtubules with occasional 4-5 nm microfilaments also detectable. The microtubules with their complement of intermediate filaments extended to the distal filopodia terminating at the secondary branches (Figures 2-16, 2-17). Figure 2-18 illustrates an example of filamentous mitochondria closely associated with numerous microtubules and IFs.

NGF-treated T9 Cells: Ultrastructural changes induced were elaborate in comparison with the changes seen in T9 control and GMF-treated cells. These changes were characterized by reduction of the nuclear size versus an extended cytoplasmic surface, increase in cytoplasmic organelles, including mitochondria, SER, RER, golgi complexes, and numerous lipid inclusions with myelin figures (Figure 2-19, 2-20). The nuclear membrane was generally smooth and occasionally interrupted by irregular indentations. Nuclear heterochromatin was well dispersed and nuclei contained usually a solitary nucleolus.

Higher magnification (Figure 2-21), revealed parallel arrays of tortuous and disoriented microfilaments intermingled with cytoplasmic organelles. The cistern of the RER and SER were closely associated with these filaments. As seen in Figure 2-22, the cytoplasm was enriched with an elaborate meshwork of slender, long microtubules and IFs which appeared to converge at the centrioles. Figure 2-23 illustrates the terminal filopodia and the secondary branches radiating in a "fern-like" manner. The body of the filopodia had numerous parallel stacks of rigid microbtubules

and IFs in close association with endoplasmic reticulum and mitochondria (Figure 2-24). This cytoplasmic framework terminated in the secondary branches of the filopodia (Figure 2-25). This well-arranged laminated cytoskeletal meshwork extending into the processes was not observed in untreated T9 cells.

Intercellular junctional complexes developed between closely opposed plasma membranes of cells in both treatments (Figures 2-26, 2-27). This occurred by fusion of the outer leaflet of the adjacent cell membranes forming a single intermediate continuous dense line which obliterated the intercellular space. A thick band of electron-dense material can be seen on both sides of the cytoplasm at the free end of the complex. The structural appearance of this junctional complex is consistent with a zonula occludens or tight junction.[23]

Figure 2-1 **Scanning electron micrograph of control anaplastic T9 glioma cells grown in HL-1 media. The cells are variable in shape and size with disorientated piling-over growth pattern. Scant microvilli are discernible along the cytoplasmic borders. (440 X).**

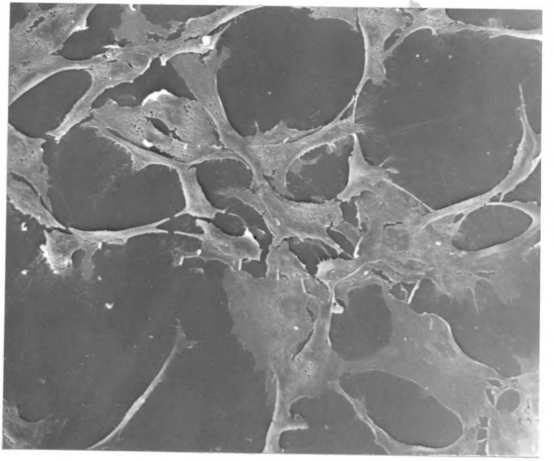

Figure 2-1

Figure 2-2 **Scanning electron micrograph of a single control T9 cell. Notice the broad multipolar cell body with thick foot processes. Variably-sized microvilli are scattered randomly over the cell body and along the cytoplasmic edges. (2200 X).**

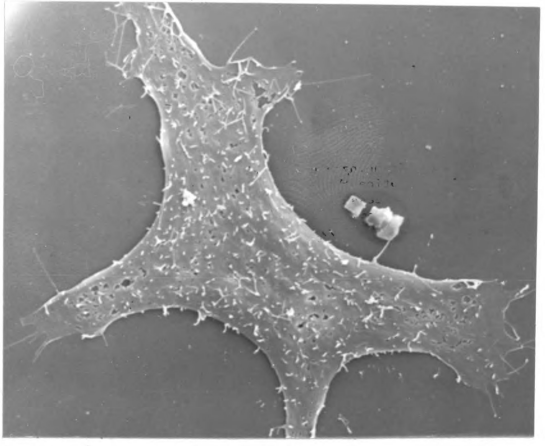

Figure 2-2

Figure 2-3 **Scanning electron micrograph. The bilayered control T9 cells have specialized surface spherical structures or bulbous excrescences called zeiotic blebs. These exocytes are associated with cells during active mitosis. (960 X)**

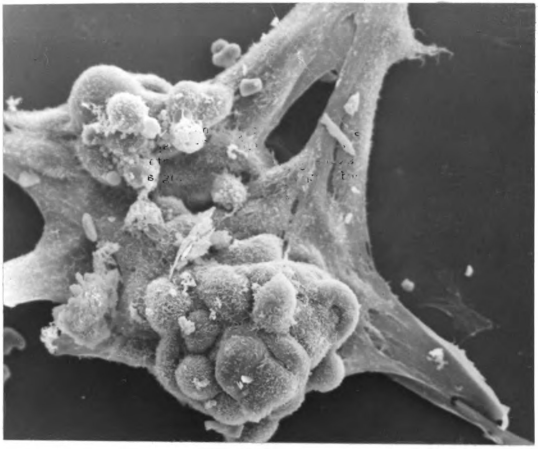

Figure 2-3

Figure 2-4 **Scanning electron micrograph of anaplastic T9 glioma cells after exposure to GMF for 4 days. Notice cells are markedly reduced in size and have long cytoplasmic processes which form an interconnecting somatic network with the processes from the neighboring cells. (440 X)**

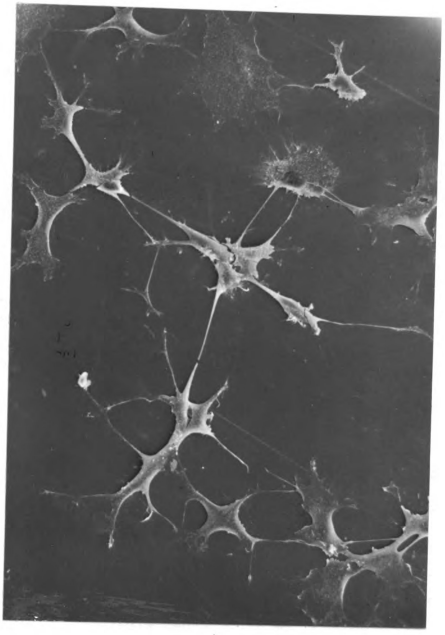

Figure 2-4

Figure 2-5 **Scanning electron micrograph of anaplastic T9 glioma cells after exposure to NGF for 4 days. Notice multipolar cells are relatively reduced in size and have broad cytoplasmic expansions or lamellopodia from which protrude extremely fine thread-like processes, filopodia. The cells are connected by broad bands of cytoplasmic projections. (X 720)**

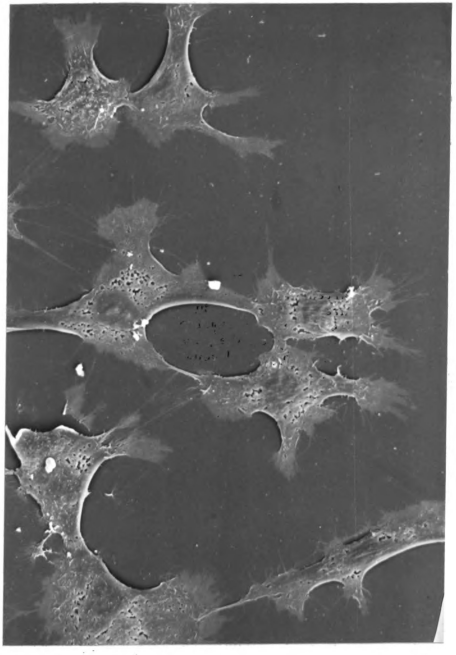

Figure 2-5

Figure 2-6 **Scanning electron micrograph. High magnification of T9 glioma cells in Figure 2-5. Cytoplasmic processes appear to blend with processes from adjacent cells forming an intricate network. The free ends of the projections have delicate expansion of lamellopodia (La) and fine filopodia (Fi). (1200 X)**

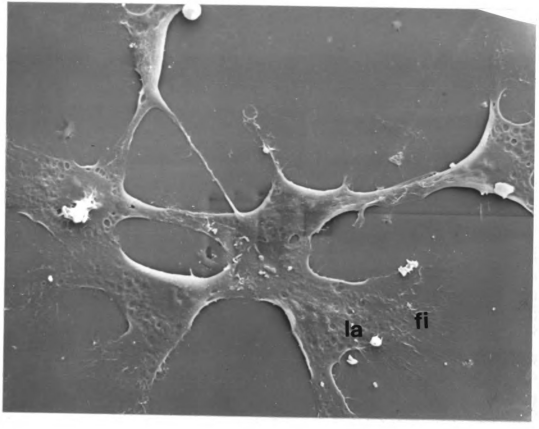

Figure 2-6

Figure 2-7 **Scanning electron micrograph of a single T9 glioma cell 4 days after exposure to NGF. Notice poles of the cytoplasmic projections are decorated with extremely fine and delicate film of lamellopodia, filopodia and secondary branches. The body surface and cytoplasmic edges are sparsely covered with microvilli. (1560 X)**

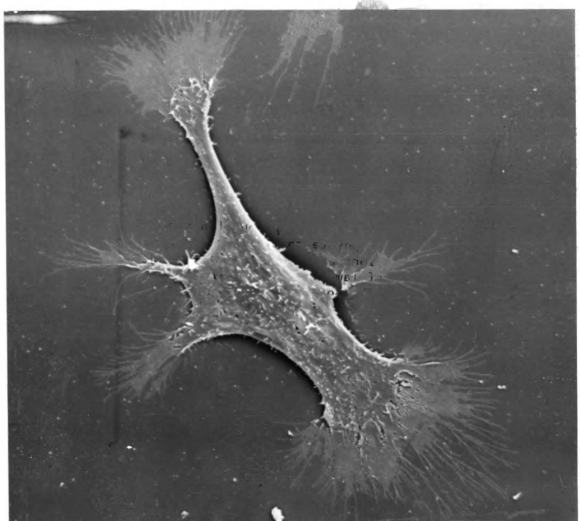

Figure 2-7

Figure 2-8 **Scanning electron micrograph. High magnification of a cytoplasmic projection of NGF-treated T9 glioma cell as seen in Figure 3-7. Notice elaborate and delicately expanded lamellopodia (La), filopodia (Fi) and secondary branches (S). (3600 X)**

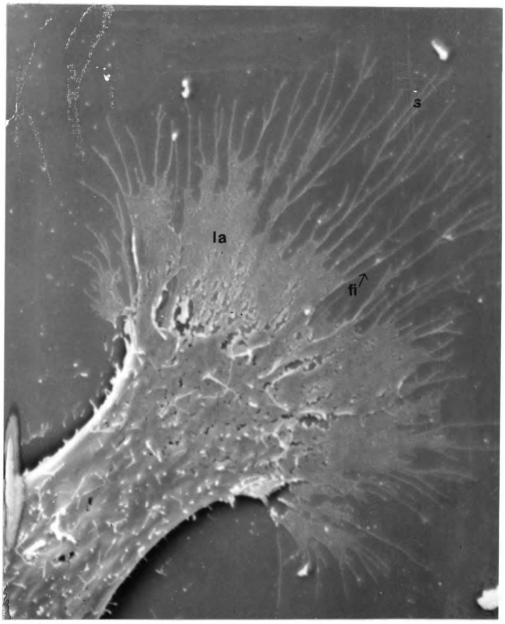

Figure 2-8

Figure 2-9 **High magnification of terminal portion of cell structure in Figure 2-8.**

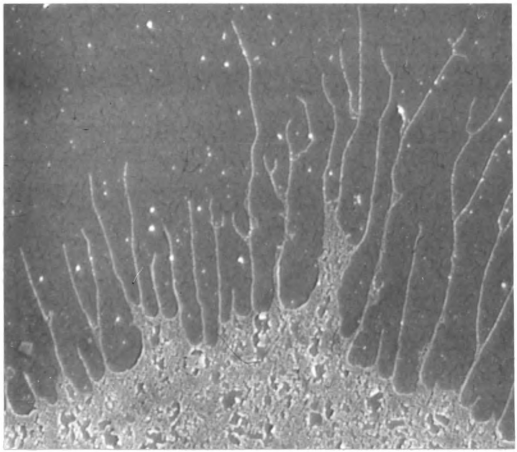

Figure 2-9

Figure 2-10 **Transmission electron micrograph of control anaplastic T9 glioma cells grown in HL-I media for 4 days. Notice the cells are large and polyhedral in shape with high nuclear: cytoplasmic ratio. The nuclear contour is rough and sometimes jagged. The nucleus contains multiple dense nucleoli. Notice in the cytoplasm there is rudimentary mitochondria, SER, RER and free ribosomes. Microvilli are rare along the cell border. (3400 X)**

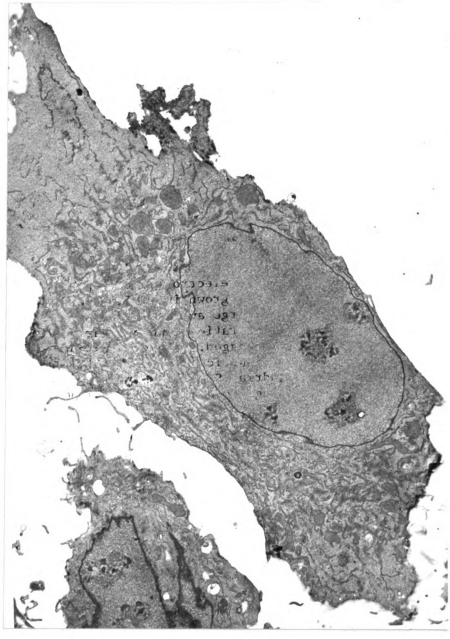

Figure 2-10

Figure 2-11 **Transmission electron micrograph. High magnification of the anaplastic T9 glioma cell in Figure 2-10. Notice in the perikaryon 5nm microfilament (mf) dispersed among RER, SER, mitochondria (m), and free ribosomes (r). n; nucleus. (34,200 X)**

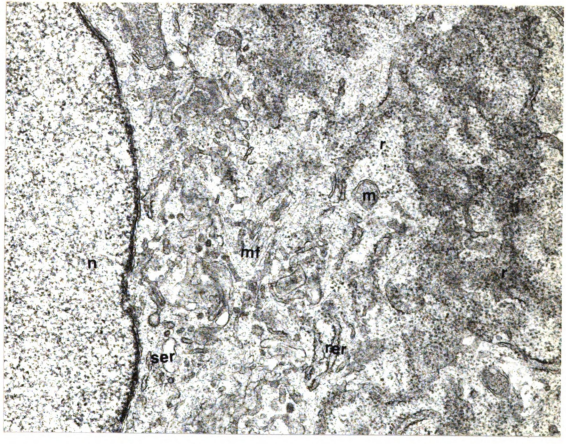

Figure 2-11

Figure 2-12 **Transmission electron micrograph of T9 glioma cell after 4 days of exposure to GMF. The cells are relatively reduced in size with low nuclear:cytoplasmic ratio. The nucleus is irregular and contains condensed marginated chromatin material. Abundant dense mitochondria, SER, RER, myelin figures, and free ribosomes are present in perikaryon. Numerous wavy microvilli project from cell edges. Notice several points of somatic attachment with the neighboring cells. (3400 X)**

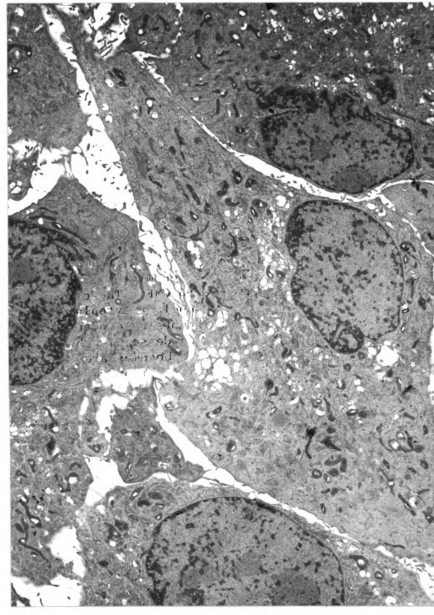

Figure 2-12

Figure 2-14 **Transmission electron micrograph of T9 glioma cells after 4 days exposure to GMF illustrates the intimate intertwining of microvilli between opposing cells. (4500 X)**

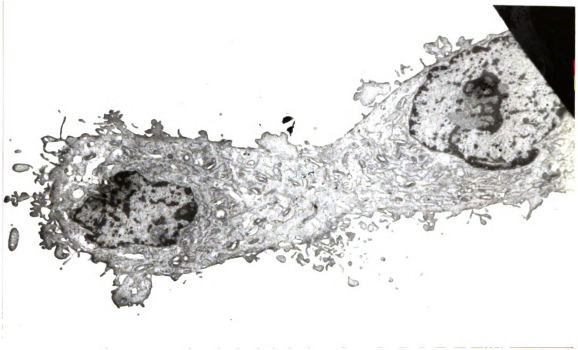

Figure 2-14

Figure 2-13 **Transmission electron micrograph of T9 glioma cells after 4 days exposure to GMF. Notice growth of fine filamentous cytoplasmic processes. (3400 X)**

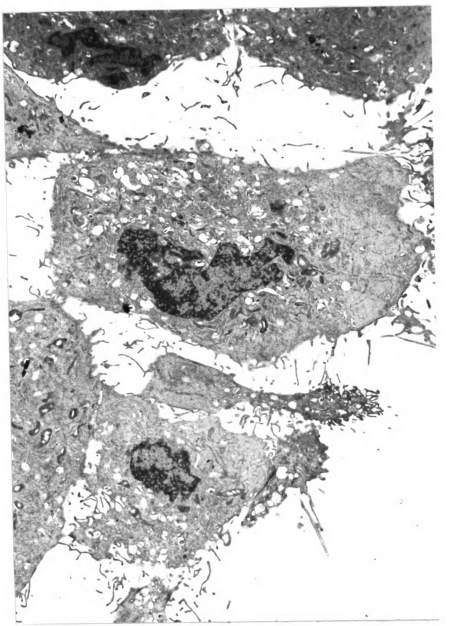

Figure 2-13

Figure 2-15 **Transmission electron micrograph. High magnification of T9 glioma cells 4 days after exposure to GMF. The perikaryon has abundant parallel rows of 25 nm microtubules (mt), 10 nm intermediate filaments (IF) and wavy bands of microfilaments (mf). These cytoskeletal frameworks are intimately associated with RER. mv; multivesicular body. (27,000 X)**

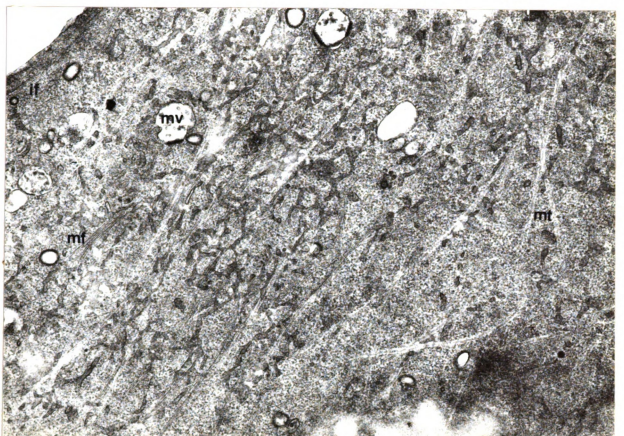

Figure 2-15

Figure 2-16 **Transmission electron micrograph of a cytoplasmic process in T9 glioma cell 4 days after exposure to GMF. Notice stacks of microtubules, intermediate filaments and microfilaments arranged along the long axis of the body of the cytoplasmic filopodia. (34,200 X)**

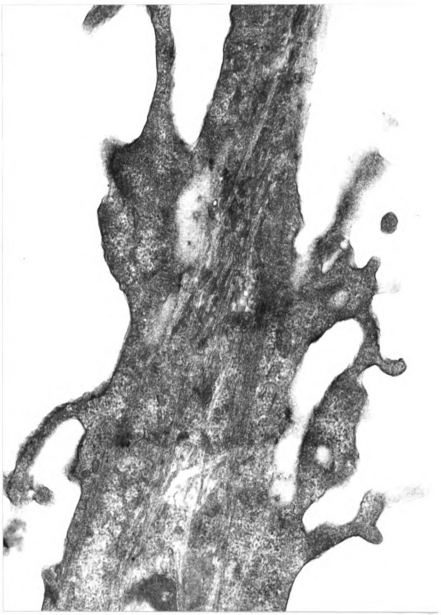

Figure 2-16

Figure 2-17 **Transmission electron micrograph of secondary branch of a filopodia in Figure 2-16. Equal complements of the cytoskeletal support extends into the terminal branches.**

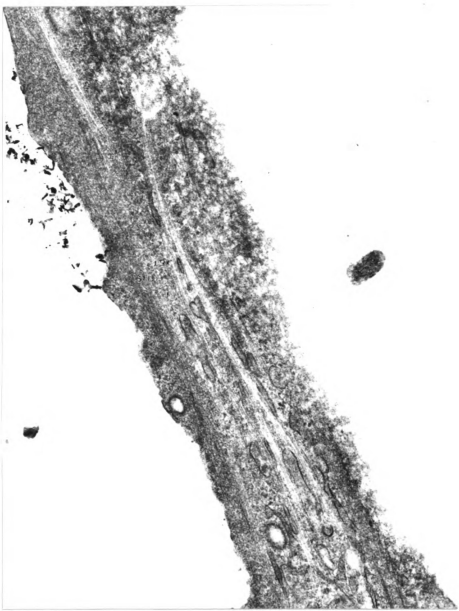

Figure 2-17

Figure 2-18 **Transmission electron micrograph. High magnification of T9 glioma cell after exposure to GMF. Notice the gigantic mitochondria are closely associated with streaks of microfilaments and intermediate filaments. (45,000 X)**

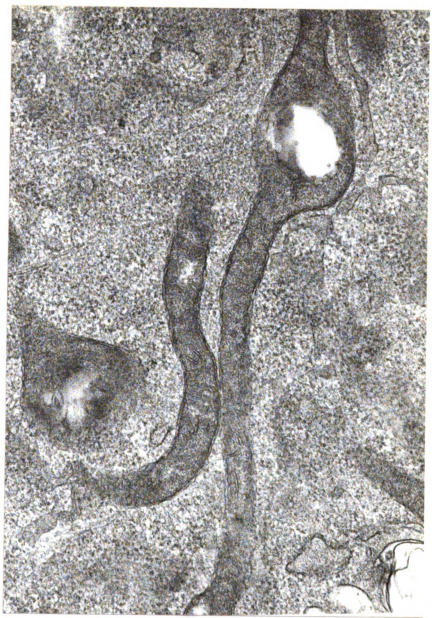

Figure 2-18

Figure 2-19 **Transmission electron micrograph of T9 glioma cell after 4 days exposure to NGF. The condensed cells have low nuclear:cytoplasmic ratio with smooth nuclear contour and evenly dispersed heterochromatin. A = elonaged mitochondria; L = lipid inclusions. (4500 X)**

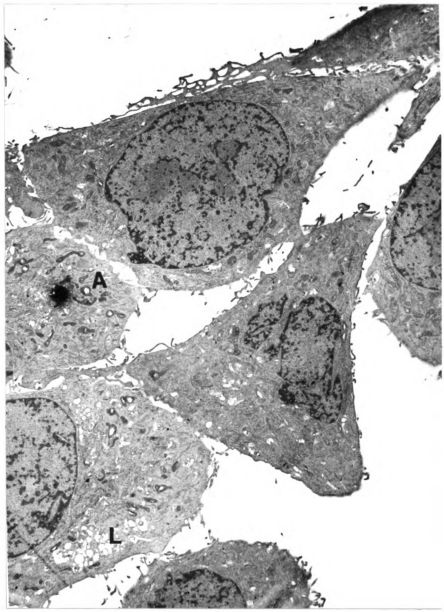

Figure 2-19

Figure 2-20 **Transmission electron micrograph of T9 glioma cell after 4 days exposure to NGF. Notice profuse growth of tortuous villi from the cytoplasmic borders (arrow). M = myelin figure; A = elongated mitochondrias; R = rough endoplasmic reticulum. (4500 X).**

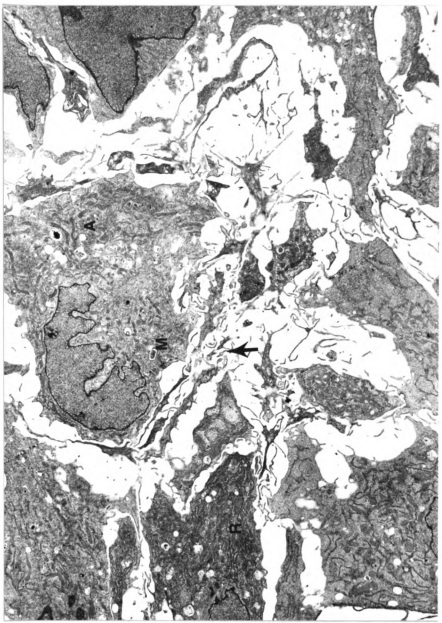

Figure 2-20

Figure 2-21 **Transmission electron micrograph of a T9 glioma cell 4 days after exposure to NGF. The perikaryon has many microfilaments (m) and intermediate filaments (f).**

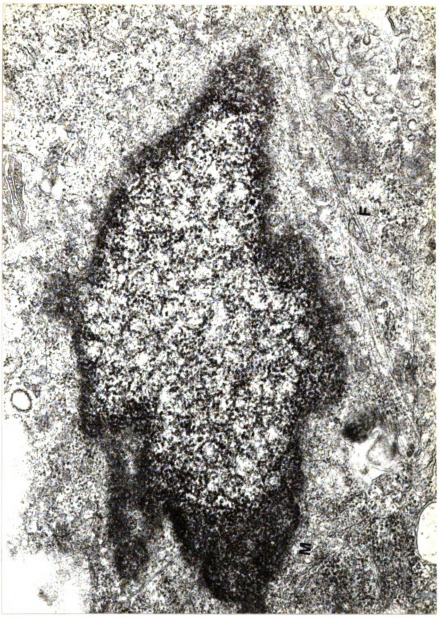

Figure 2-21

Figure 2-22 **Transmission electron micrograph of T9 glioma cells after exposure to NGF. Notice streaks of microfilaments, intermediate filaments and microtubules converge around the two centrioles (c). R = rough endoplasmic reticulum; G = dilated golgi apparatus. (19,800 X)**

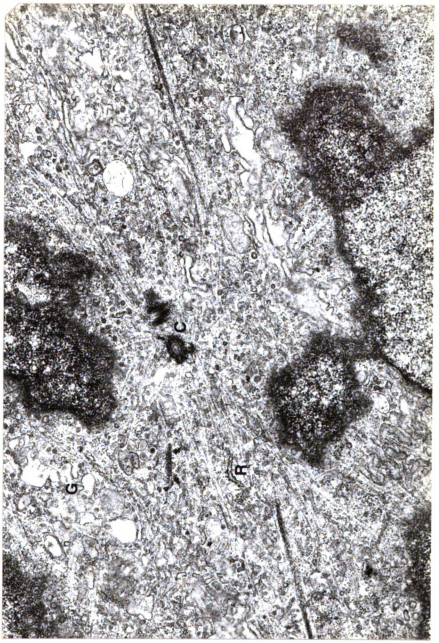

Figure 2-22

Figure 2-23 **Transmission electron micrograph of T9 glioma cells after exposure to NGF. The figure illustrates terminal portion of a cytoplasmic projection (P), lamellopodia (L), and filopodia (F) corresponding to the SEM details of cells in Figure 2-8. (3420 X)**

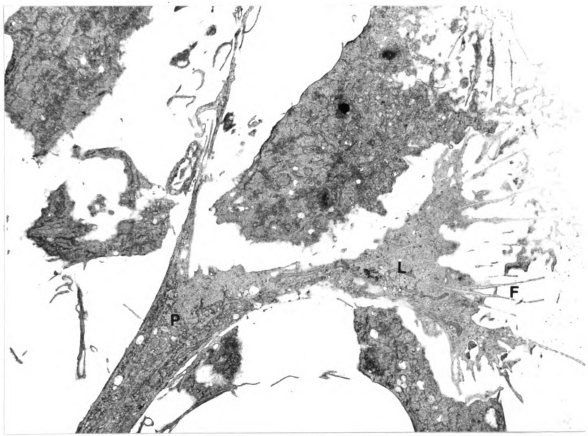

Figure 2-23

Figure 2-24 **Transmission electron micrograph. High magnification of cytoplasmic projection (P) as in Figure 2-21. The long axis of the process is enriched with parallel bundles of microfilaments, intermediate filaments and microtubules. Note that these cytoskeletal frameworks are in close association with rough endoplasmic reticulum and mitochondria. (45,000 X)**

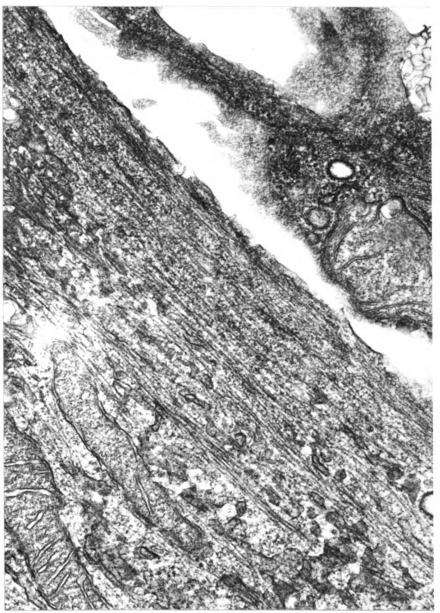

Figure , 2-24

Figure 2-25 **Transmission electron micrograph. High magnification of filopodium as in Figure 2-21. The process contains rich complement of structural support.**

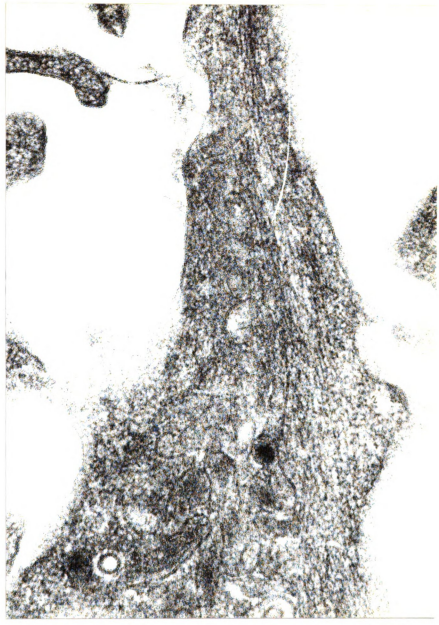

Figure 2-25

Figures 2-26 2-27 **Transmission electron micrograph of T9 glioma cells after exposure to NGF (Figure 2-26) and GMF (Figure 2-27). Note the junctional complex, zonula occludens (JC) between two opposing cells. The outer leaflets of the opposing cell membrane form a single intermediate dense line. (81,000 X)**

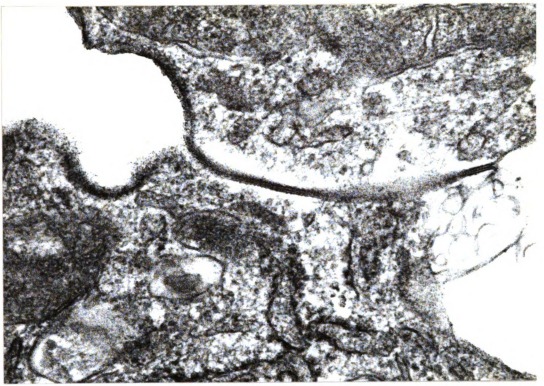

Figure 2-26

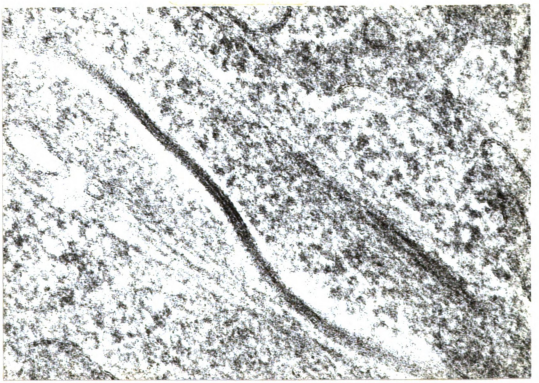

Figure 2-27

Discussion

This study revealed several cell surface and internal ultrastructural changes induced in T9 anaplastic glioma cell line after treatment with NGF and GMF. While the internal fine components promoted by both these growth factors are essentially identical, the cell surface features are characteristically different. The T9 cells attained a cylindrical and multipolar appearance with reduced cell size after exposure to GMF. Long cytoplasmic processes or filopodia had a tendency to make connections or blend with the processes from other cells. The observed surface morphological alterations following GMF treatment was similar to glioblast maturation as studied by Lim et al.[24]

NGF promoted a totally different cell surface feature with flattened, broad cell bodies with "foot" processes ramified with weblike fine branches. This modification of phenotypic expression may be a form of cell maturation or differentiation process unique to NGF influence on the T9 cell line.

Greater evidence for cell maturation in the present study is the detailed revelation of the internal cell elements by TEM. Glia maturation factor and NGF promoted extension of elaborate cytoskeletal structures, notably the assembly of microfilaments, IFs and microtubules in the long axis of the cytoplasmic processes. This finding is consistent with other studies,[26-28] in which it was shown that exposure of ganglion cells to NGF promoted neurite outgrowth and concurrent assembly of cytoplasmic neurofilaments and microtubules forming the basis of the structural support in the treated cells.

GMF may also stimulate the differentiation of glioblasts and activate the synthesis of these structural elements.[24] The concentration as well as the integrity of microtubules are essential for the morphological differentiation of cells (Lim[25]). The appearance of a distended golgi apparatus, fat globules, abundant SER, RER, free ribosomes, and the increased number and size, as well as distribution of mitochondria are all indicative of cell differentiation.

The importance of cell junctions between adjacent cells may be viewed as indication of a shift towards a higher state of cell maturation.[23,29] It is believed that these junctions may contribute to contact inhibition of cell growth.[30] Malignant cells, on the other hand, lack contact inhibition which has been linked to the absence or defects in cell junction communication.[31] In the present study, cell junctions (zonula occludens) between opposing NGF- and GMF-treated cells were prominent features.

The NGF-mediated action is an event which follows NGF interaction with its receptor binding sites on responsive cells (Chapter II). It is logical, therefore, to assume that the effects of NGF and GMF on the T9 glioma cell were induced via a similar receptor mediated action. Studies[31,32] have elucidated that, following the protein-receptor binding, biochemical events start at the level of the cell membrane, in the cytoplasm and in the nucleus. This molecular mechanism of mediation involves secondary messengers such as cyclic AMP and Ca^{+2}.[34] The overall effects of these growth factors in the process of maturation of transformed cells may be conceived as a modulation or regulation of gene expression through an epigenetic mechanism.

It should be of great interest to investigate the possibility of cell-cell communication through gap-junction channels in the differentiated cells. Equally rewarding would be an *in vivo* experiment in which the animals could be inoculated with treated and untreated cells to investigate the histologic appearance of the tumor cells in a clinical environment.

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CHAPTER 4

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF CENTRAL AND PERIPHERAL NERVE TUMORS INDUCED BY ETHYLNITROSOUREA IN RATS UTILIZING ANTI-GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP), ANTI-LEU 7, AND ANTI-S-100 PROTEIN ANTIBODIES

SUMMARY:

N-nitrosourea-induced central and peripheral nerve tumors in Sprague-Dawley rats were tested for immunoreactivity with glial fibrillary acidic protein (GFAP), S-100 protein and human natural killer-1 (HNK-1, also called Leu-7) isotope antibodies. The avidin-biotin-complex (ABC) method for GFAP and S-100, and the unlabeled antibody immunoperoxidase (peroxidase-antiperoxidase, PAP) method for HNK-1 were used.

In peripheral nerve neurinomas, S-100 immunoreactivity varied from strongly positive in differentiated tumor cells to weakly positive in anaplastic neurinomas. None of the neurinomas, irrespective of the degree of differentiation, reacted to GFAP and HNK-1 antibodies.

In CNS tumors, S-100 and GFAP were reliable markers for astrocytomas and were especially useful in characterizing the astrocytic cells within mixed gliomas, a feature which is not readily demonstrable with routine hematoxylin and eosin stain preparations. The perikaryon and processes of reactive astrocytes showed intense positivity with GFAP and S-100 protein compared to normal and neoplastic counterparts. Oligodendrogliomas were consistently negative for GFAP and S-100, except for 3 out of 36 that showed a superficial rim of weak reaction with S-100 protein antibody. S-100 expression was demonstrable in meningiomas and glioependymomas. The HNK-1 antibody stain was not useful in our investigation.

INTRODUCTION

Prior to the advent of immunohistochemistry, the standard method for characterizing tumors included light and electron microscopic studies. Immunochemistry provided an exciting new diagnostic tool for distinguishing tumors of diverse histogenetic origin.[1-4] The principle is based on the demonstration of antigen in tissue samples utilizing poly or monoclonal antibodies produced selectively against the cell antigenic markers. In neuro-oncology, immunohistochemistry has tremendous benefits when used in adjunct with conventional procedures. The nervous system is composed of a variety of cell types. In order to understand the behavior and management of the tumors arising from different cell precursors, it is extremely important to accurately identify the major tumor cell component and also characterize the degree of anaplasia.

The mammalian nervous tissue cytoskeleton consists of intermediate filaments (IF), microfilaments and microtubules. Among the IF are the neurofilaments, glial fibrillary acidic protein (GFAP), and vimentin. While the former is the principal constituent of IF in the neurons,[5-8] of the latter two, GFAP is the predominant IF in the glial cells.[9-10]

Antibodies against GFAP are commonly used to characterize normal, reactive and neoplastic cells of astroglial origin.[9-11] Varying degrees of immunoreactivity to GFAP have been demonstrated in rat and human Schwann cells,[6,12,13] human ependymal cells,[12,13] rat enteric glial cells,[14] tumors of human peripheral nerve sheath,[11,15,16] and rat sensory and sympathetic ganglia satellite cells.[15] Similar reactions have been demonstrated in non-nervous tissues such as in human

salivary gland adenomas[17] and human epiglottis cartilage.[18] In neuro-oncology, demonstration of GFAP has several distinct objectives:

- to identify astrocytes and tumors arising from glial cells.
- to distinguish between glial and non-glial tumors.
- to demonstrate astrocytic components in mixed CNS tumors such as glioependymomas.
- to identify tumor cells outside the CNS, i.e., expression of GFAP may indicate the metastatic nature of astroglial tumors.

The next useful antigenic marker is the S-100 protein.[19] It was first isolated as a highly acidic soluble protein from rabbit and bovine nervous systems.[20,21] The compound is synthesized in glial cells,[22,23] and Schwann cells,[24] but its precise function is not known. Almost all cells of necroectodermal origin can be immunohistochemically shown to contain varying amounts of S-100 protein.[25-28] However, S-100 immunoreactivity is not restricted to the cells of the nervous system, but a diverse spectrum of mammalian tissues have been demonstrated to express this protein.[29-32] Despite the wide range of distribution, S-100 is considered a valuable cell marker for normal, reactive and neoplastic tissue of neuroectodermal origin.[19,30]

Another useful method of cell identification can be accomplished by the use of monoclonal antibodies (MAB) raised against specific cell-surface antigenic determinants or epitopes. One such MAB is anti-HNK-1, also known as anti-Leu 7, originally raised against the human T-lymphoblastoid cell line, which has been reported to recognize antigens on human cells with natural killer cell activity.[32] Subsequent work has revealed that this MAB cross-reacts with a range of human and rodent tissues,

including the elements of the nervous system.[33-39] The advantage of using HNK-1 in determining tumors of central and peripheral nervous systems is based on the principle that this MAB recognizes a single specific cell surface antigen.[31] Although a high percentage of oligodendrogliomas display HNK-1 positivity, this immunostain cannot be considered a specific markerfor oligodendrogliomas, since other neuroepithelial tumors also react with this MAB.[39]

In one other study,[40] the cellular morphology of ENU-induced rat brain microtumors were compared with those in macrotumors by determining the levels of GFAP and Leu 7 in both neoplasms. While the microtumors were found to be negative for both these stains, macrotumors were positive either for GFAP or Leu 7. The aim of the present test was to characterize, utilizing the HNK-1 MAB and antibodies against GFAP and S-100 protein with immunoperoxidase method, all neurogenic tumors obtained from rats after exposure to ENU, and also, to determine the relationship, if any, between the degree of anaplasia or the stage of differentiation and intensity of the reaction by these antibodies.

MATERIALS AND METHODS

Tumors

ENU-induced tumors of the central (CNS) and peripheral (PNS) nervous systems in Sprague-Dawley rats (described in Chapter II) were investigated. Samples were fixed in 10% buffered formalin and embedded in paraffin. Several 5 um thick sections were cut, and one section of each tumor was routinely stained with hematolylin and eosin for histopathological classification. The remaining sections were used for immunohistochemical study.

Immunohistochemistry

Glial Fibrillary Acidic Protein (GFAP)

In order to prevent detachment of the tissue sections from the glass slides during trypsinization (see below), the glass slides were first coated with poly-L-lysine[a] (25 mg/200 ml distilled water). The avidin-biotin-complex (ABC) method[41] for immunoperoxidase stain was used. A commercially available ABC kit, Vectastain,[b] was obtained from Vector Laboratories.

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[a] Sigma, St. Louis, Missouri

[b] Vector Laboratories, Inc., Burlingame, California

Procedure

(1) Sections were deparaffinized in xylene for 5 minutes and rehydrated through graded ethyl alcohol concentrations to distilled water for 3 minutes at each change.

(2) After a rinse with phosphate buffer saline (PBS), tissues were digested for 3 minutes in prewarmed (37°C) 0.1% trypsin, washed in distilled water, and dipped in PBS for 3 minutes.

(3) Two drops of 3% hydrogen peroxide (H_2O_2 in methanol) was added for 5 minutes to the sections to block the endogenous peroxide activity.

(4) Sections were thoroughly rinsed for 3 minutes in two changes of PBS, and all further incubations were carried out in a humidifier at room temperature.

(5) Two to three drops of normal goat serum was applied for 30 minutes.

(6) After tapping off excess serum, the sections were treated with 2-3 drops of 1/300 anti-rabbit $GFAP^c$ for 30 minutes.

(7) Sections were rinsed for 30 minutes in two changes of PBS and then incubated with 2 drops of biotinylated antibody (goat-anti-rabbit IgG) for 30 minutes.

(8) Following two washings in PBS, sections were exposed to 2 drops of avidin-biotinylated conjugate (ABC) for 30 minutes.

(9) The sections were rinsed in 2 changes of PBS, and the reaction was developed in freshly prepared 3.3'-diaminobenzidine tetrahydrochloride (DAB) for 10 minutes.

(10) After a brief wash in deionized water, the sections were treated with copper sulfate solution to enhance the DAB development, then rinsed in deionized water and counter-stained with Gill's hematoxylin for 30 seconds.

(11) Finally, the sections were dehydrated in graded alcohol, cleaned in xylene and mounted in permount mounting medium. Positive controls stained in parallel included human cerebral cortex (obtained during autopsy) and rat brain.

S-100 Protein Stain - ABC Vectastain[b]

All sections were immunostained using anti-S-100 protein antibody.[b] The ABC method employed was essentially identical to that applied for GFAP test, except that in step 6, bovine anti-rabbit S-100 antibody (1/200) was substituted for 30 minutes. Positive controls stained in parallel consisted of human normal skin, human cutaneous melanoma, human cerebral cortex (obtained during autopsy), and rat brain.

Human Natural Killer - 1 Monoclonal Antibody (HNK-1 MAB)

Sections were immunostained with HNK-1 MAB, commercially available as Anti-Leu 7.[d] The following 4-step peroxidase-anti-peroxidase (PAP) method was based on the procedure previously described.[42,43]

PAP

(1) After the sections were deparaffinized in xylene for 10 minutes and delipidized in chloroform for 30 hours, they were rehydrated through graded methyl alcohol (95% and 70%) to distilled water for 3 minutes at each change.

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[c] Dako Corporation, Santa Barbara, California

[d] Becton and Dickson, Mountain View, California

(2) Sections were treated for 30 minutes with hydrogen peroxide (H_2O_2 in methanol) to block endogenous peroxidase activity.

(3) After a 5 minute wash in PBS, sections were exposed to dilute normal rabbit serum,[c] (1% in PBS) for 20 minutes to inhibit non-specific binding of immunoglobulins.

(4) Without rinsing, the sections were incubated overnight at 4°C with Anti-Leu 7 diluted 1:300 in PBS.

(5) After washing in buffer for 5 minutes, they were incubated in link antibody[c] for 20 minutes (rabbit anti-mouse immunoglobulin).

(6) Sections were washed in PBS before incubation for 1 hour in peroxidase-anti-peroxidase complex.[c]

(7) After washing for 5 minutes in PBS, sections were immersed in freshly prepared 3.3'-diaminobenzidine tetrahydrochloride (DAB)[a] for 10 minutes.

(8) The sections were washed in deionized distilled water, counter stained with Gill's hematoxylin (1 minute), dehydrated in graded series of ethanol, cleaned in xylene, and mounted in permount mounting media.

PREPARATION OF REAGENTS

PBS (phosphate buffered saline) pH 7.4

Sodium chloride (NaCl).....16 gm

Sodium phosphate dibasic anhydrous (Na_2HPO_4).....2.5 gm

Potassium phosphate, monobasic anhydrous (KH_2PO_4).....0.4 gm

Potassium chloride (KCl).....0.4 gm

Triple distilled water.....2000 ml

Trypsin Solution

PBS, prewarmed 37°C.....200 ml

Trypsin (Sigma-type II from porcine pancreas)..... 0.5 gm

Diaminobenzidine-Hydrogen Peroxide Substrate Solution

3.3' Diaminobenzidine tetrahydrochloride (DAB).....5 mg

PBS.....10 ml

3% Hydrogen peroxide (H_2O_2)..... 3 drops

RESULTS

Neurinomas

S-100 protein: All 55 peripheral nerve neurinomas examined showed positive stain for S-100 protein, although the intensity of the immunostain indicated an inverse relation with the degree of malignancy. Figures 3-1 and 3-2 illustrate differentiated trigeminal nerve neurinomas with a majority of the tumor cells expressing S-100 protein. Intense positive reaction was generally concentrated in the cell membranes, in cytoplasm and nucleus. A clear halo was sometimes evident between the strongly positive cell membranes and the nuclei. On the other hand, anaplastic or undifferentiated neurinomas had scattered nests of cells that showed mild to weakly positive stain, while a majority of the anaplastic cells were negative.

GFAP: In contrast to S-100 protein reaction, none of the 55 neurinomas had any evidence of immunoreactivity against the GFAP antibodies. Figure 3-3 shows a serial section of the neurinoma in Figure 3-1 immunostained for GFAP. Note that the section is at the junction of CNS and PNS (trigeminal nerve). The strongly positive GFAP stain is confined to the astroglial cells and their processes in the CNS, providing a distinct demarcation from the GFAP negative trigeminal nerve neurinoma.

Astrocytomas

S-100: Of the 12 astrocytomas, 9 well-differentiated and 3 moderately undifferentiated astrocytomas stained positively for S-100 protein. Figure 3-4 illustrates a typical differentiated astrocytoma in which a majority of neoplastic cells show strong positive reaction on the cell membranes as well as in the cytoplasm, while the thin cytoplasmic

processes stain granular. The staining intensity and the number of positive cells were not determined by the degree of the tumor cell differentiation.

GFAP: The expression of GFAP in astrocytomas was identical to that of S-100 protein. The reactive astroglial cells within and peripheral to the tumor stained more intensely than the actual neoplastic cells and the normal counterpart. As depicted in Figure 3-5, GFAP positive tumor cells in a differentiated cerebral astrocytoma have strong reactivity in the cytoplasm and processes.

The 3 undifferentiated astrocytomas contained scattered individual positive staining cells (Figure 3-6). However, the tumor border was speckled with characteristically strongly immunostained reactive astrocytes.

Oligodendrogliomas

S-100: Of the 36 oligodendrogliomas, there was weak positive staining of 3 and all others were negative. As indicated in Figure 3-7, the small oligodendrocytes have a thin rim of cell membrane showing positivity. However, the bulk of the tumor-supporting framework is composed of fibrous processes of reactive fibrillary astrocytes. The cytoplasm and broad processes of these cells stained intensely positive.

GFAP: All oligodendrogliomas were negative for GFAP. However, there was positive staining reactive fibrillary astrocytes within the tumors. Figure 3-8 illustrates the densely stained, large, multipolar reactive cells and the tortuous fibrillary processes stretched between the tumor cells. These stellate cells were randomly scattered throughout the tumors.

Mixed Gliomas

In 20 mixed gliomas (astrocytes-oligodendrocytes), both S-100 and GFAP stained intensely positive in neoplastic or reactive astrocytes. Figures 3-9 and 3-10 depict a typical mixed glioma in which astroglial cells were fairly evenly spread amongst the small oligodendroglial cells. These stellate cells had abundant cytoplasm and often coarse tapering cell processes, consistent with astrocytes. The oligodendroglial cells were consistently negative in all cases.

Glioependymomas

The ependymal component showed a positive reaction for GFAP and S-100 protein. Figure 3-11 represents a glioependymoma stained for S-100 protein. Note there are several clusters of variably-sized positive, large, ependymomal cells. In Figure 3-12, GFAP positive ependymomal cells have a delicate fibrillary network radiating from the cytoplasm.

Meningiomas

None of the 4 meningiomas tested stained positive for GFAP. All 4 had positive staining of variable intensity for S-100. As can be seen in Figure 3-13, a majority of meningioma cells in the vicinity of the blood vessels show varying degrees of immunostain. The negatively stained erythrocytes and vascular endothelial cells serve as contrast.

Anti-Leu 7 (Anti-HNK-1)

Despite several attempts, specimens of all CNS and PNS tumors tested had negative staining with anti-Leu 7. This was felt to be due to an expression of presumably different isotopes in rat cells (Rubinstein, personal communication) not recognized by the Anti-Leu 7 antibodies.

Figure 3-1 **Photomicrograph of a trigeminal nerve neurinoma immunostained for S-100 protein. CNS-PNS junction (arrow). Immunoperoxidase, ABC method; Hematoxylin counter stain. 400 X.**

Figure 3-2 **Higher magnification of trigeminal nerve neurinoma in Figure 3-1. Notice positive reaction on cell membranes, in cytoplasms and nuclei. 640 X.**

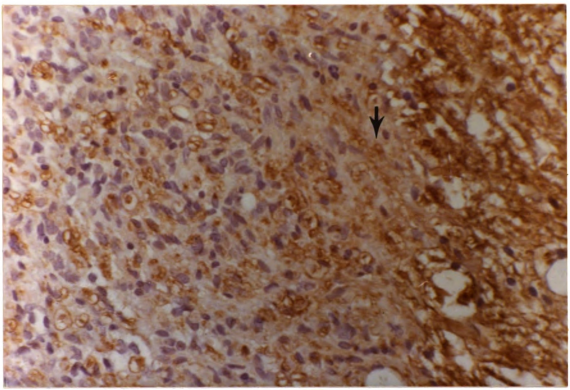

Figure 3-1

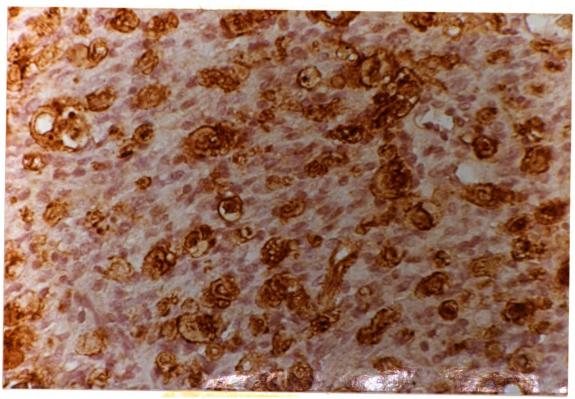

Figure 3-2

Figure 3-3 **Photomicrograph of trigeminal nerve neurinoma immunostained for GFAP. The neurinoma cells are negative, whereas the astrocytes in the CNS are strongly positive. CNS-PNS junction (arrow). Immunoperoxidase, ABC method. Hematoxylin counterstain. 400 X.**

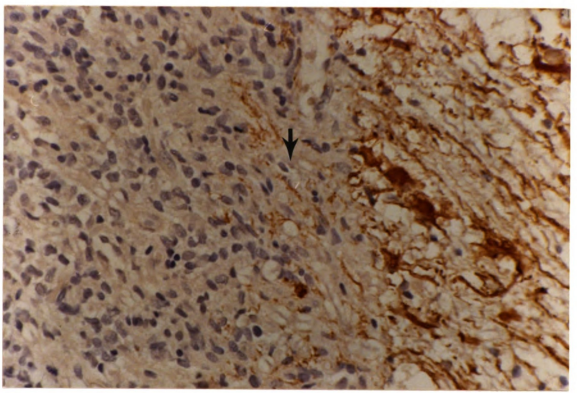

Figure 3-3

Figure 3-4 **Photomicrograph of an astrocytoma immunostained for S-100. Immunoperoxidase, ABC method. Hematoxylin counterstain. 400 X.**

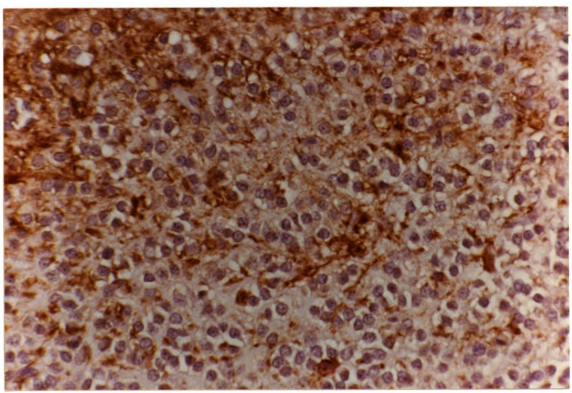

Figure 3-4

Figure 3-5 **Photomicrograph of a cerebral astrocytoma immunostained for GFAP. Immunoperoxidase ABC method. Hematoxylin counterstain. 400 X**

Figure 3-6 **Photomicrograph of a cerebral undifferentiated astrocytoma immunostained for GFAP. Notice peripheral large reactive astrocytes stained intensely while an occasional astrocytoma cell within the tumor showed a weak reaction. Immunoperoxidase, ABC method. Hematoxylin counterstain. 160 X.**

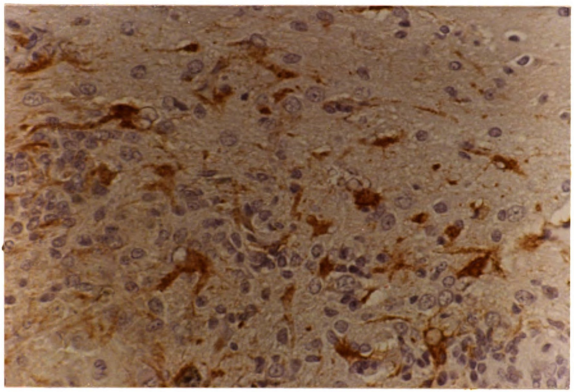

Figure 3-5

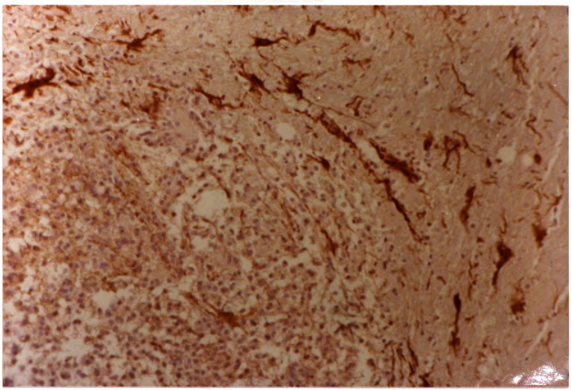

Figure 3-6

Figure 3-7 **Photomicrograph of an oligodendroglioma immunostained for S-100. Notice strong positive reaction in the cytoplasm and processes of reactive astrocytes within the tumor. The small oligodendroglioma cells showed weak positive reaction (arrow). Reactive fibrillary astrocytes (arrowhead). Immunoperoxidase, ABC method. Hematoxylin counterstain. 400 X.**

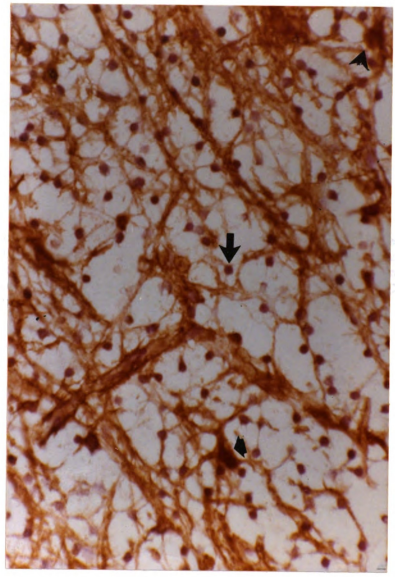

Figure 3-7

Figure 3-8 **Photomicrograph of an oligodendroglioma immunostained for GFAP. Notice intense positive reactions in the reactive fibrillary astrocyte within the tumor. The oligodendroglials are negative for GFAP. Immunoperoxidase, ABC method. Hematoxylin counterstain. 400 X.**

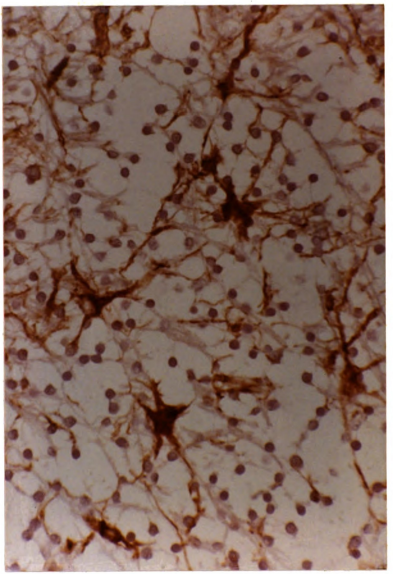

Figure 3-8

Figure 3-9 **Photomicrograph of a mixed glioma immunostained for S100. Notice strong positive reaction in the broad astrocytic cytoplasm and its thin processes. The oligodendroglioma cells do not stain for S100. Immunoperoxidase, ABC method. Hematoxylin counterstain. 400 X.**

Figure 3-10 **Photomicrograph of a mixed glioma immunostained for GFAP. The astrocytic component of the tumor shows strong positive reaction, whereas the oligodendroglioma cells are uniformly negative. Immunoperoxidase, ABC method. Hematoxylin counterstain. 400 X.**

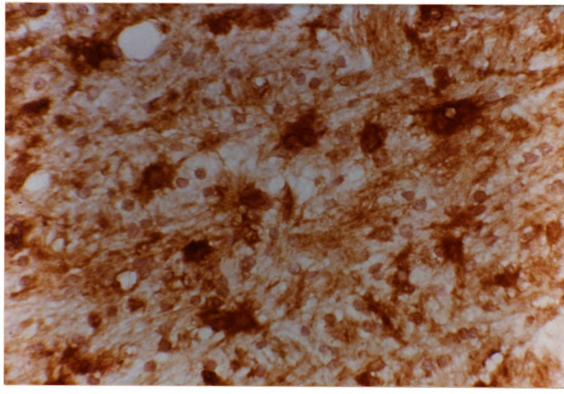

Figure 3-9

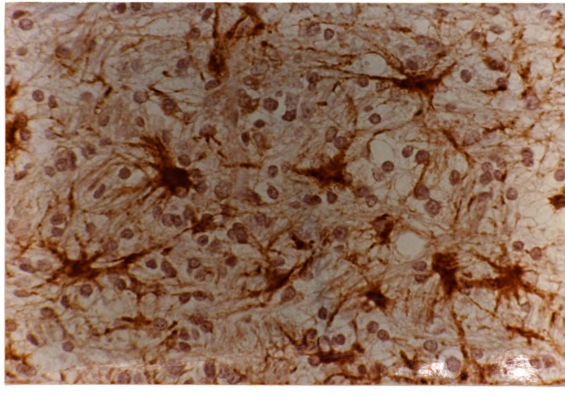

Figure 3-10

Figure 3-11 **Photomicrograph of a glioependymoma immunostained for S-100. Notice many positive cells forming the pseudo-rosettes. Immunoperoxidase, ABC method. Hematoxylin counterstain. 400 X.**

Figure 3-12 **Photomicrograph of a glioependymoma immunostaind for GFAP. Notice several tumor cells have positive stain in the thin membraneous cell processes. Immunoperoxidase, ABC method. Hematoxylin counterstain. 400 X**

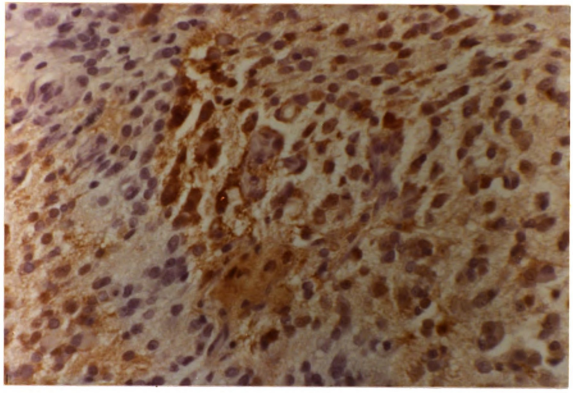

Figure 3-11

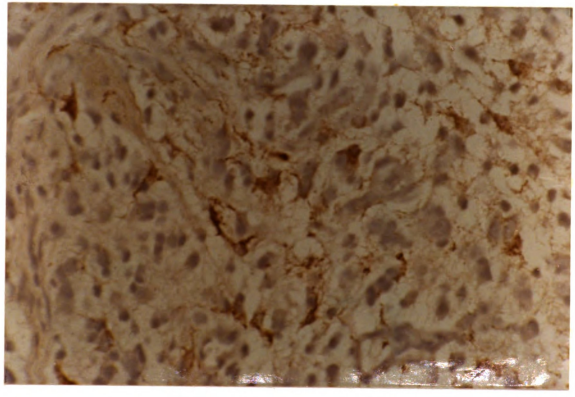

Figure 3-12

Figure 3-13 **Photomicrograph of a meningioma immunostained for S100. Notice relatively strong reaction in cells surrounding the blood vessels. Immunoperoxidase, ABC method. Hematoxylin counterstain. 400 X.**

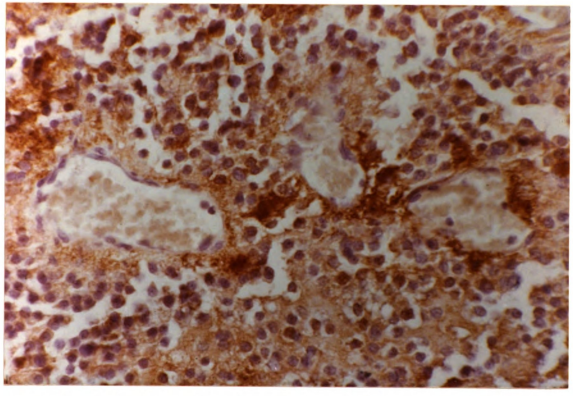

Figure 3-13

DISCUSSION

Glial fibrillary acidic protein, S-100 and Leu 7 antibodies are a few amongst a battery of immunohistochemical stains utilized in the differential diagnosis of naturally-occurring neurogenic neoplasms. These staining procedures have been specifically helpful in cases of doubtful or questionable tumor cell origin or tumor cell composition.

The present work utilizing experimentally induced intra- and extracranial neurogenic tumors in rats discloses significant information in respect to the three cell markers employed.

(1) The expression of intermediate filaments (GFAP) and S-100 protein correlated with the degree of tumor cell differentiation.

(2) Although GFAP and S-100 protein antibodies are reliable markers of carcinogen-induced neurogenic tumors, the anti-Leu 7 antibodies utilized did not recognize rat neurogenic tumor cells.

(3) Tumors suspected of mixed cell composition can be characteristically identified by the use of these immunostains.

In peripheral nerve neurinomas, it was evident that S-100 protein was the most reliable cell marker, although the immunostaining intensity and the number of positive cells decreased with increasing degree of tumor anaplasia. The correlation between malignancy and expression of S-100 protein in carcinogen-induced neurinomas was not determined; however, it can be hypothesized that malignant cells which resemble primitive embryonal stem cells are less endowed with essential molecules necessary for the expression of the protein. Contrary to reports[13,17,18] of the occurrence of GFAP positive cells in neurinomas, in the present study, neither the differentiated nor the anaplastic carcinogen-induced

neurinomas showed any evidence of GFAP reaction. The presence of GFAP in normal and neoplastic Schwann cells is controversial. It has been suggested that a subpopulation of Schwann cells with cytoplasmic 10 nm intermediate filaments could cross-react with GFAP antisera, and this could account for the report of GFAP positive cells in neurinomas.[43]

The most consistent immunochemical reaction with GFAP and S-100 was observed in astrocytomas. Although the number of undifferentiated astrocytomas tested was small (3), immunoreactivity with S-100 was not affected by the degree of tumor anaplasia. Our test results indicated that the number of GFAP positive cells and staining intensity of positive cells was lower in undifferentiated astrocytoma cells when compared to the differentiated neoplastic cells. This is consistent with previous reports[2,13,48] that the expression of GFAP in astrocytoma cells decreases with increasing cellular atypia. Since GFAP is an indication of astrocyte maturation (or differentiation), its reported absence in anaplastic tumor cells and progenitor cells may be viewed as an inability of less differentiated cells to produce GFAP. Thus, the significance of GFAP staining in different types of astrocytomas is controversial. Tascos *et al*[13] documented that all astrocytomas react positively with GFAP, although the staining intensity and the number of positive cells decreased with advancing stages of tumor anaplasia. For example, gemistocytic astrocytes stained most intensely, followed by poorly differentiated astrocytoma cells (grade III-IV) where only isolated cells were positive. In glioblastoma multiforme, only the gemistocytic and multinucleated giant cell components showed varying degrees of reaction. However, two recent studies[43,49] showed that all astrocytomas had strong GFAP stain, regardless of their degree of malignancy.

Both GFAP and S-100 stains proved useful in the case of mixed gliomas by staining the astroglial and not the oligodendroglial components of the tumor justifying the classification. It must be emphasized that small numbers of astrocytes can normally be present in oligodendrogliomas either as peripheral reactive cells or cells entrapped in the tumor (Figure 3-6), features that may not be readily detectable in routine hematoxylin and eosin preparations.

The expression of GFAP and S-100 in glioependymoma, and the presence of S-100 positive cells in meningioma observed in our study are in agreement with others.[13,43] The meningioma cells in the vicinity of the blood vessels exhibited a high degree of S-100 positive reaction. In glioependymoma, S-100 and GFAP expression were detected in the cytoplasm and along the processes of cells forming the pseudorosettes.

Contrary to a report[42] of strong S-100 immunoreactivity of oligodendrogliomas, in our study only 3 of the 36 oligodendrogliomas showed superficial positive reaction. Our experience was also in disagreement with reports[12,49] of positive GFAP in oligodendrogliomas. The expression of GFAP in oligodendrocytes has been controversial. Some authors[4,13,48] believe oligodendrogliomas are GFAP negative, while others[12,43,50] described GFAP immunoreactivity in these tumor cells. However, it was not conclusively determined whether the positive cells in the above studies were true neoplastic oligodendroglial cells or small gemistocytic astrocytes, which are normal components of most oligodendrogliomas. It may also indicate that the positive cells represent a totally different class of glia termed gliofibrillary oligodendrocytes which, during development, can transiently express GFAP.[50]

Results of our investigation differ remarkably in some areas from those obtained with human tumor studies. Studies involving specimens derived from diverse genetic origin (human versus animal), natural versus experimentally induced tumors and laboratory test procedures could potentially contribute to inconsistent results. Despite these reservations, the present study further confirmed that GFAP and S-100 protein antibodies have useful application in diagnostic pathology. This investigation was particularly valuable in characterizing rat astrocytomas and components of mixed gliomas. Although the study was limited to 3 immunohistochemical stains, it has set a strong basis for testing of inconclusive and/or tumors of suspected neurogenic origin by including a wider spectrum of cell "markers" such as vimentin, neuron specific enolase (NSE), myelin basic protein (MBP), carbonic anhydrase C (CA C) and neurofilaments (NF).

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