Effect of Nerve Growth Factor on the Transplacental Induction of Neurinomas by Ethylnitrosourea in Sprague-Dawley Rats

Narayan R. Raju, Adalbert Koestner, Keiji Marushige, Kathryn L. Lovell, and Dorothy Okazaki

Department of Pathology, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Administration of nerve growth factor (NGF) to the offspring of Sprague-Dawley rats transplacentally exposed to 50 mg/kg ethylnitrosourea on the 20th day of gestation resulted in a significant reduction of trigeminal and peripheral nerve neurinomas. Forty, 60, and 80 μg of NGF was administered in five s.c. doses, one dose on each of days 12–16, 90–94, and 210–214 postnatally. Of the 34 rats in the NGF-treated group, 11 animals were affected with trigeminal nerve neurinomas as compared to 18/34 in the NGF-untreated group (P < 0.05). In the peripheral nerves (spinal cord nerve roots) there were five and 11 neurinomas, respectively, in each group of 34 rats. 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 was P < 0.01. Five trigeminal and two peripheral neurinomas in the NGF-untreated group were shown by immunohistochemical staining to contain nerve growth factor receptor protein, whereas none of the neurinomas in the NGF-treated group were 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 ethylnitrosourea exposure perhaps by the process of maturation and/or differentiation of the transformed cells, and that this effect may depend upon the presence of receptor binding sites.

INTRODUCTION

NGF is a polypeptide composed of α, γ, and β subunits, the latter being the active fraction (1, 2). NGF is essential for the development, survival, and regeneration of sympathetic and sensory neurons (3–6). Reports indicate that NGF has a neurotrophic effect on the cholinergic neurons in the brain (7–9). The initial event in NGF’s mechanism of action is the binding of NGF to specific plasma membrane NGFR (10, 11). Following this binding, several biochemical changes have been shown to occur in pheochromocytoma (PC 12) cells and in the cells of the superior cervical ganglia. These changes include an increase in cyclic AMP levels and mobilization of intracellular Ca2+ (12), phospholipid methylation (13), phosphatidylinositol alteration (14, 15), and an increase in cyclic AMP and Ca2+/phospholipid-dependent protein kinase (16).

A single dose of 50 mg/kg ENU given to pregnant female rats via the lateral tail vein on the 20th day of gestation results in the production of neurogenic tumors in nearly 100% of the offspring (17, 18). Sequential evaluation of these neoplasms indicate that anaplastic neurinomas (schwannomas) of the trigeminal nerves are evident as early as 20 days after exposure and by 90 days nearly 100% of the rats are affected (19). In this context the expression “neurinoma” is preferred over schwannoma since there is no documentation that anaplastic neurinomas consist exclusively of a clonal expression of Schwann cells, although electron microscopy revealed that a majority of the cells in the tumor resembled immature Schwann cells (18). The lesions include ENP in the early stage, microtumors in the intermediate stage (90-day post-ENU exposure), and grossly detectable macrotumors in the late stage (7-month post-ENU exposure). The tumors of the peripheral nerves (spinal cord nerve roots) begin to appear during the late stage of tumor development (19).

Several investigations demonstrated that NGF had both maturation and differentiation influences on neuroectodermal tumor cells (20–24). In two studies (25, 26), it was shown that treatment of pregnant rats with NGF prior to ENU exposure, or treatment of offspring postnatally following transplacental ENU administration, resulted in a significant reduction of the ENPs in the trigeminal nerve of the offspring by 90 days of age. The objective of the present experiment was to determine the persistence of the effect of NGF upon the neurinoma development over a 12-month period and to investigate whether the suppressive effect of NGF on the ENU-transformed cells was dependent upon the presence of NGFR.

MATERIALS AND METHODS

Preparation of NGF. β-NGF was isolated from the salivary glands of male Swiss Webster mice and purified by the procedure described by Bocchini and Angelletti (27). Samples were tested for biological activity by the PC 12 method (28).

Animal Experiment. Eight date-mated pregnant Sprague-Dawley (CD) rats (Charles River Laboratories, Portage, MI) were exposed to a single dose of 50 mg/kg body weight ENU via the lateral tail vein as previously described (18). The nursing rats along with the pups were randomly divided into two groups.

Group A. Thirty-four offspring (18 males and 16 females) received doses of 40, 60, and finally 80 μg of NGF (dissolved in sterile 0.15 M NaCl solution) divided in five s.c. doses on days 12–16, 90–94, and 210–214 postpartum. This method of NGF administration was chosen in order to facilitate a slow absorption and to ensure a prolonged action of NGF on the ENU-transformed neural crest cells. The incremental dose levels (20 μg) between treatment allowed for the increase in age and body weight of the rats.

Group B. Thirty-four offspring (15 males and 19 females), which served as positive controls, were exposed to ENU but did not receive NGF. They were maintained under the same conditions as the experimental group.

Termination of the experiment at 1 year of age precluded any significant natural occurrence of neurogenic tumors in CD rats (29). An NGF control was therefore not considered to be needed. Rats either died as a consequence of neoplasias or were euthanized due to progressive neurological signs and weight loss. All animals were necropsied as soon as possible after death or euthanasia. All trigeminal nerves and body weight of the rats.

Histopathology. Formalin-fixed samples were routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with H&E.

Detection of NGFR. Cryostat sections were fixed rapidly in 1% glutaraldehyde-phosphate buffer (pH 7.4) containing 1% paraformaldehyde and 2.5% sucrose. Sections were then washed in phosphate-buffered saline and equilibrated in 0.2 M sodium phosphate buffer (pH 7.4) containing 0.2 M sucrose. Sections were then incubated with goat anti-NGFR antibody (Santa Cruz Biotechnology) for 30 min at room temperature, washed in phosphate-buffered saline, and then incubated with biotinylated goat anti-goat antibody (Vector Laboratories) for 15 min at room temperature. Sections were then incubated with streptavidin-peroxidase complex (Vector Laboratories) for 15 min at room temperature, washed in phosphate-buffered saline, and then incubated with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) and hydrogen peroxide for 15 min at room temperature. Sections were then washed in phosphate-buffered saline and stained with hematoxylin.

Received 11/4/88; revised 9/5/89; accepted 9/12/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Cancer Institute Grant CA-32594.
2 A recipient of The Upjohn Company and Pruess Foundation Fellowships.
3 To whom requests for reprints should be addressed, at Monsanto Environmental Health Laboratory, 645 South Newstead Avenue, St. Louis, MO 63110.
4 The abbreviations used are: NGF, nerve growth factor; NGFR, nerve growth factor receptor; ENU, ethylnitrosourea; ENP, early neoplastic proliferation.
NGF ON NEURINOMA DEVELOPMENT

parafomaldehyde prior to exposure to the NGFR monoclonal antibody 192-IgG (30). The antibody was used at a concentration of 5 µg/ml in 100 mM potassium phosphate (pH 7.5)/160 mM NaCl/5% heat-inactivated horse serum/0.02% NaN₃ (from the laboratory of Dr. Eugene M. Johnson, Jr., Washington University Medical School, St. Louis, MO). After binding for 30 min, sections were washed in phosphate-buffered saline (20 mM potassium phosphate/150 mM NaCl, pH 7.5) and incubated for 30 min with biotinylated horse anti-mouse IgG immunoglobulin (1 µg/ml). The sections were washed and treated for 30 min with diure hydrogen peroxide in methanol (0.3% H₂O₂/methanol). This was followed by a wash and incubation with a complex of avidin and biotinylated horseradish peroxidase (Vector Laboratories, Burlingame, CA). Finally, the sections were washed and incubated for 10 min in 0.05% 3,3-diaminobenzidine/0.01% H₂O₂. Sections were rinsed in water, counterstained with hematoxylin, dehydrated, and mounted.

NGFR Positive Control. The NGFR-positive control was developed in 7-day transplanted sciatic nerve from male Sprague-Dawley rats according to the method previously described (31).

Statistical Analysis. A chi-square test of independence was used to determine if there was a significant difference between the NGF-treated rats (Group A) and the control rats (Group B).

RESULTS

In the NGF-untreated group (positive control) of 34 offspring transplacentally exposed to 50 mg/kg on day 20 of gestation, 15 rats had grossly visible neurinomas and three rats had microscopic ENPs of the trigeminal nerve (Table 1). A majority of the trigeminal nerves were involved bilaterally with gross lesions varying from edematous and unevenly swollen foci to friable, reddish-brown hemorrhagic tumors. Histologically, the neurinomas consisted of cells with hyperchromatic oval nuclei, dense chromatin, and pale cytoplasm. These cells were arranged in undulating sheets, whorls, and dense clusters (Fig. 1A). Mitotic figures varied from one to two per high power field. All neurinomas in both groups were anaplastic tumors. This is consistent with past experience with transplacental ENU exposure to tumors in the offspring (18).

The ENPs were classified according to the criteria previously described (18, 19). These foci were most often located in the proximity of the junction between peripheral nervous system and central nervous system, and were characterized by disorganized arrays of hyperchromatic cells haphazardly arranged in irregular sheets (Fig. 1B). In the NGF-treated group, 10 of the 34 offspring had trigeminal nerve neurinomas and one offspring had an ENP (Table 1). This is in contrast to 15 neurinomas and three ENPs in the untreated group. The data indicate that treatment with NGF after transplacental ENU exposure resulted in a significant reduction in the number of trigeminal nerve neurinomas (P < 0.05). When the total numbers of neurinomas (trigeminal and spinal nerve roots) between the groups are compared, the effect of NGF on the reduction of neurinoma development is highly significant (P < 0.01, Table 1).

To determine if the effect of NGF observed in this study was dependent upon the presence of NGF, 45 neurinomas from both groups were examined by NGFR by the immunoperoxidase method. Five trigeminal and two peripheral nerve neurinomas (7/29) were positive for NGFR (Fig. 2A) in the control group while none of the 16 neurinomas in the NGF-treated group were positive. The NGFR positive and negative controls are illustrated in Fig. 2A and B.

DISCUSSION

The results of this study provide evidence that NGF is capable of reducing neurinoma development in rats transplacentally exposed to ENU. It complements and extends the previous 90-day studies (25, 26) by establishing the persistence of the neurinoma-reducing effect recognized in those experiments.

Since the mechanism of NGF interaction with the neurons is dependent upon the presence of NGFRs (10, 11), the effect of NGF on the ENU-initiated neuroepithelial cells is also expected to be dependent upon the binding of the hormone to the receptor molecules present on neurinoma cells. In the present study, NGFR protein was present only in the NGF-untreated group and none of the 16 neurinomas in the NGF-treated group were positive. These results support the theory that the seven neurinomas of the untreated group which were positive for the NGFR protein would have been eliminated had these rats been treated with NGF. It was surprising that only 25% of the rats in the NGF-untreated group had demonstrable NGF receptors. One would assume that the difference between that number and that of the rats responding to NGF in group A is just an expected biological variation, or the tumor cell population changed with time and age, so that cells either failed to synthesize the receptor protein or receptor-containing cells were out-numbered and overgrown by others. This study suggests the hypothesis that NGF binding to receptor sites and consequential internalization results in growth retardation and differentiation (maturation) of anaplastic tumor cells rendering them

| Table 1 Effect of NGF on peripheral nerve neurinoma development occurring after ENU administration. Group A, NGF-treated; Group B, NGF-untreated |
|---|---|---|---|---|---|
| Group | No. of rats | No. of trigeminal nerve neurinomas | No. of peripheral nerve neurinomas | Total neurinomas | Age Range (Average) |
| A | 34 | 11 (32%)<sup>a</sup> | 5 (15%) | 16 (47%)<sup>b</sup> | 160–347 (243) |
| B | 34 | 18 (53%)<sup>a</sup> | 11 (32%) | 29 (85%)<sup>b</sup> | 182–316 (233) |

<sup>a</sup> P < 0.05.  
<sup>b</sup> P < 0.01.

Fig. 1. A, photomicrograph of an anaplastic neurinoma. Notice hyperchromatic neoplastic cells have dense nuclei, pale cytoplasm, and mitotic figures (arrows). H&E, x 400. B, photomicrograph of an ENP characterized by marked hypercellularity in a trigeminal nerve. Arrows, junction between CNS and PNS. H&E, x 100.
subject to the normal control mechanisms of the body (death and elimination). A recently completed study in our laboratory demonstrated the potential of NGF as a reverse-transformation agent in vitro (32). Treatment of anaplastic glioma cell with NGF permanently modulated the growth and morphological characteristics of undifferentiated neoplastic glial cells. The effects included an arrest of cell multiplication and induction of phenotypical changes such that the cell ultrastructurally resembled differentiated astroglial cells. Similar effects were also obtained with anaplastic neurinoma cells.

Since NGF was administered in the present study at three stages of tumor development it is not possible at this time to separate the effects of NGF upon susceptible neurogenic tumors at various developmental stages. It will therefore be necessary in the future experiments to distinguish the effects of NGF on early tumor development from its effect upon macroscopically recognizable neurogenic tumors. Knowledge of the latter would be of more predictive value for human glioma therapy in which the presence of NGF receptors had been established on biopsy materials of tumors.

ACKNOWLEDGMENTS

We are thankful to Dr. Eugene M. Johnson, Jr., Washington University Medical School, St. Louis, MO, for providing the NGFR monoclonal antibody 192-IgG and for his useful suggestions during the experiments.

REFERENCES


23. Reynolds, C. P., and Perez-Polo, J. R. Induction of neurite outgrowth in the...
NGF ON NEURINOMA DEVELOPMENT


Effect of Nerve Growth Factor on the Transplacental Induction of Neurinomas by Ethylnitrosourea in Sprague-Dawley Rats


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/49/24_Part_1/7120

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.