Neuronal Growth Factors from Tumours of Von Recklinghausen Neurofibromatosis

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ABSTRACT: Explants of 21 neurofibromas from 16 patients with Von Recklinghausen neurofibromatosis (NF-I) plus tumour tissue from 5 comparison patients and normal tissue from one of the NF-I patients were assayed for neuronal growth factor(s) using dissociated embryonic sensory neurons from chick embryo. Twenty-one of 21 neurofibroma explants released detectable quantities of neuronal growth factors, but only 2 of 8 non-neurofibroma tissue explants released activity.

While antiserum to mouse nerve growth factor (NGF) fractionally inhibited neurite outgrowth induced by some of the neurofibromas, overall differences between assays containing antibody and controls reached statistical significance in 3 cases; in one case, explants of a separate tumour from the same patient had no detectable NGF-like activity.

These data support the hypothesis that local release of neuronal growth factors in neurofibromas are responsible for neurites observed within these tumours. Further evidence that endoneurial tissue of peripheral nerve is a rich source of heterogeneous neuronal growth facts has been provided by these studies.

RÉSUMÉ: Facteur de croissance neurotrope extrait de tumeurs provenant de patients souffrant de neurofibromatose de von Recklinghausen Au moyen de neurones sensitifs embryonnaires dissociés provenant d'embryons de poulet, nous avons dosé le facteur de croissance neurotrope dans des explants de 21 neurofibromes provenant de 16 patients atteints de neurofibromatose de von Recklinghausen (NF-I), ainsi que dans du tissu tumoral provenant de 5 patients contrôles et dans du tissu normal provenant d'un des patients avec NF-I. Vingt-et-un des 21 explants neurofibromateux libéraient des quantités détectables de facteur de croissance neurotrope. Cependant, seulement 2 des 8 explants non-neurofibromateux libéraient cette substance active.

Bien que l'antisérum dirigé contre le facteur de croissance neurotrope (FCN) inhibait de façon fractionnaire les excroissances des neurites induites par certains des neurofibromes, dans l'ensemble, les différences entre les dosages dans les expériences avec anticorps et les expériences contrôles étaient statistiquement significatives dans 3 cas; dans un cas, des explants d'une autre tumeur provenant du même patient n'avaient pas d'activité analogue au FCN détectable.

Ces données sont en faveur de l'hypothèse selon laquelle la libération locale de facteurs de croissance neurotropes dans les neurofibromes est responsable des neurites observés à l'intérieur de ces tumeurs. Ces études ont fourni d'autres preuves que le tissue de l'endonèvre des nerfs périphériques est riche en facteurs de croissance neurotropes hétérogènes.

Can. J. Neurol. Sci. 1987; 14:141-144

Neurofibromatosis (NF) is a heterogeneous disease which includes Von Recklinghausen neurofibromatosis (NF-I). NF-I is distinguished from other forms of NF on the basis of large numbers of cafe-au-lait spots, cutaneous neurofibromas, and iris Lisch nodules; these findings are often associated with other features as reviewed by Riccardi.[1]

The influence of nerve growth factor on cells of neural crest origin and the presumed crest derivation of NF lesions have stimulated a number of studies[2-7] of circulating NGF levels in NF in order to establish a reliable laboratory marker for neurofibromatosis. We have demonstrated recently that human serum contains neuronal growth factors but that the level of the factors does not contribute to identification of the NF-I phenotype.[8]

Despite compelling evidence that peripheral nerve endoneurium contains both NGF and non-NGF material,[9,10,11] little attention has been devoted to a study of neuronal growth factors in the peripheral nerve tumours of NF patients. Using a sensitive neuronal cell biological assay,[12] we report here detection of neuronal growth factors from explants of tumours of a rela-

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Received September 24, 1986. Accepted January 6, 1987

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tively homogeneous group of NF-*1* patients.

METHODS

Tissue Explants Tissue collected by one of us (VMR) between 1982 and 1984 was harvested at the time of surgery and frozen immediately at −80°C. Material was stored at this temperature until used in the assays. At the time of assay 1 mm^3 explants were prepared aseptically with a scalpel using pieces of tissue that appeared on gross examination to be homogeneous. Mean (±S.D.) protein per explant analyzed by the method of Lowry et al[13] was 163±71μg. A single 1 mm^3 piece of tissue was added to each cultural well. For each assay at least two explants of tissue were analyzed, and a minimum of four separate assays were carried out for each tissue.

Bioassay

The bioassay was based upon a chick embryo single sensory neuron culture system developed as a sensitive biological assay for NGF.[12,14] In the assay, culture wells of Falcon microculture plates were coated with poly-D-lysine (0.1mg/ml, 24 h, 37°C). Following washes of the wells, a suspension of 10,000 7- to 8-day dorsal root ganglion cells enriched for neurons by preplating was added to each well in a total volume of 200 μl of supplemented Ham's F12 medium[12] with 5% fetal calf serum. A single 1 mm^3 explant of tissue was added to each well. Control wells contained NGF (4 pM) or no NGF. In addition, each well contained 5 μl heat-inactivated (56°C, 30 min) rabbit anti-NGF antiserum (final concentration 1/200), or 5 μl of a similar dilution of heat-inactivated pre-bleed normal rabbit serum (NRS). Antiserum concentration was 1000-fold in excess of that required to completely inhibit neurite outgrowth produced by NGF (4 pM).

Culture plates were incubated for 24 h (37°C, 5% CO_2) and then scored for percent neurite-bearing cells. At least one hundred phase bright cells in a representative field of each well were counted using a Leitz Diavert microscope with phase optics. This assay measures numbers of neurons with processes in excess of 1.5 cell diameters but does not quantitate process length, another parameter that can be used for neurotrophic activity. One of us (RJR) scored all assays without knowledge of the source of tissue. Results were matched to the tissue of origin by VMR.

MATERIALS

NGF was prepared using the method of Mobley et al.[15] Rabbit antimouse NGF was prepared as previously described.[8] Commercial sources were used to purchase all chemicals and media: trypsin (TRPCK) from Worthington Biochemical Corporation; Ham's F12, Hank's balanced salt solution, fetal calf serum from GIBCO; poly-D-lysine from SIGMA; carboxymethyl cellulose (CM52) from Whatman; ampholytes from LKB; gel electrophoresis materials from BioRad; fertilized White Leghorn eggs were purchased from H&M Poultry and Waterfowl and incubated in a home-made egg incubator.

RESULTS

The single neuronal cell assay reliably gave background neurite outgrowth of 5±5% in the absence of NGF and values of 67±9% (Mean ± SD) in the presence of 4 pM NGF. These data are consistent with findings of the dose response of sensory neurons to NGF where, between 3 and 4 pM, approximately 70% of neurons bear processes at 24 to 48 h.[14] In the assay of explants, data were expressed as percent neurite outgrowth per 1 mm^3 explant both in the presence of antiserum to NGF and in the presence of prebleed normal rabbit serum at the same dilution. Control wells in the absence of rabbit serum were not different from wells containing normal rabbit serum (data not shown).

Table 1 indicates that 21 of 21 tumours from 16 patients contained detectable neuronal growth factor activity (Mean ± SD, n=8). In four cases, percent neurite outgrowth was in excess of that observed with the maximal response to NGF. This observation, and the finding that neurite outgrowth was inhibited only partially by anti-NGF, is evidence of the heterogeneity of the explant-released activity. Table 1 also shows the neuronal response in the presence of anti-NGF. Overall, the inhibition by anti-NGF suggesting the presence of growth fac-

Table 1. Neuronal Growth Factors from Tissue Explants.

Patient	Specimen	% Neurite-bearing Cells Control	Anti-NGF
1	nf	90.2±12.2	70.3±11.6**
	nf	81.5±11.9	79.0± 4.9
2	nf	62.3±28.6	62.5±20.6
3	nf	14.1±11.3	12.8±16.8
4	nf	76.9±15.4	64.5±15.6
5	nf	48.5±21.3	39.8±12.0
6	nf	54.7±10.9	47.5± 6.4
7	nf	33.3± 4.0	41.5±16.0
8	nf	39.3±15.4	21.8± 8.3
9	nf	34.7±22.5	45.3± 8.7
	nf	33.1±19.5	30.5±23.1
10	nf	56.6±30.8	34.3±15.2
	nf	29.5±22.8	31.1±22.5
11	nf	64.3±14.9	44.0± 7.1*
12	nf	51.7±17.5	61.8±16,3
13	nf	66.7± 7.9	67.3±16.0
	nf	27.5±22.9	35.8±25.9
	nf	46.9±28.2	46.8±23.9
14	nf	40.3±23.7	43.9±20.6
15	nf	14.1± 8.7	13.0±12.1
16	nf	71.7±13.2	51.8±18.4***
13	liv	0§	0
17	men	36.3± 4.8	27.5± 5.0
18	wt	0	0
	wt	0	0
	wt	0	0
19	wt	19.7±20.9	24.9±27.0
20	nb	0	0
21	nb	0	0

* p<.05
** p<.02
*** p<.01
§ % neurite-bearing cells was 5% or less.

nf neurofibroma
wt Wilm's tumour
nb neuroblastoma
men meningioma
liv liver

Cultures of neurons were prepared as described in Materials and Methods. At 24 hours, culture wells were scored for the percentage of viable cells with processes exceeding 1.5 cell diameters. Results are expressed as means and standard deviations of assays set up in duplicate and carried out four times.

https://doi.org/10.1017/S0317167100026263 Published online by Cambridge University Press

tor immunologically cross-reactive with mouse NGF was not significant. However, with three neurofibroma explants, there was a statistically significant inhibition, suggesting the presence of a NGF-like molecule.

Comparisons for this study were comprised of seven tumours from five patients, and normal tissue from one patient with NF-I who provided three neurofibromas for assay. Of the comparison tumours, one of four Wilm's tumours had low but detectable activity, while no activity from either of two neuroblastomas could be detected. One meningioma had levels of activity comparable to some of the neurofibromas. The normal tissue, liver from a patient with neurofibromatosis, had no detectable activity.

Discussion

In recent studies of serum neuronal growth factors in Von Recklinghausen neurofibromatosis[8] and in the present studies, the problem of clinical heterogeneity that has made interpretation of previously reported biological data difficult has been minimized by using material from well-characterized NF-I patients. As in the serum studies, we have employed a biological assay system which minimized the contribution of contaminating non-neuronal cells that are a source of neuronal growth factor activity.[14,16] Furthermore, it has been possible, using the sensory neuronal cell assay, to detect growth factor heterogeneity and immunological homology of tissue-released NGF-like activity with NGF of murine origin.[9,14]

The present studies provide semi-quantitative data demonstrating that 100% of NF-I tumours tested released neuronal growth factors. In only three cases, however, did anti-NGF significantly inhibit the neurite-promoting activity released by the tumours, suggesting the presence of molecular species homologous to mouse NGF. The percent inhibition of neuronal performance by anti-NGF in three tumours averaged 27 percent. This figure is consistent with the percent inhibition by anti-NGF of neuronal growth factor activity from explants of peripheral nerve *in vitro* in two previous studies.[9,10] It is of interest that in one instance another neurofibroma from the same patient released neurite-promoting material with no detectable NGF-like activity; this observation suggests that autologous neurofibromas could behave independently. One tumour in the comparison group of tumours — a meningioma — had levels of neuronal growth factor activity comparable to that released by some neurofibromas. The other tumours and normal tissue that were tested had little or no activity. It is possible that the higher levels in the meningioma relates to its presumptive embryonic origin from neural crest structures,[17] but two undifferentiated neuroblastomas, also of the same embryonic origin,[18] had no activity.

The wide variability of the assay scores in the presence of explants contrasts with the highly reproducible values seen with NGF. This observation might suggest different kinetics of release of neuronal growth factors from individual tissue explants. More precise quantitation with less variance might have been achieved by using extracts of tumour homogenates, but wide variability was also observed when serum extracts were assayed for similar activity in a recent study.[8]

The present observations that neurofibromas are a rich source of neuronal growth factor activity support the hypothesis advanced previously[8,18] that local release of growth-promoting materials is responsible for neurites observed within neurofibromas.[19] Previous studies[8] demonstrating that serum levels of neuronal growth factors in NF-I patients do not distinguish this phenotype from comparison subjects suggests that the tumour activity is not released to the general circulation. The present data also add further support to previous observations that both NGF and non-NGF neuronal growth factors are produced by endoneurial cells of peripheral nerve.[9,10,11]

Important goals of future research will be the characterization of non-NGF neuronal growth factors in peripheral nerve. Neurofibromas are a readily available source of such material and may also be a rich depot of those molecular species that are involved in proliferation of endoneurial elements within the tumours.

Acknowledgements

This work was supported by the Physicians' Services Incorporated Foundation of Ontario (RJR) and the Texas Neurofibromatosis Foundation (VMR). The excellent technical assistance of S. Faulkner is acknowledged. The authors are grateful to Mrs. S. McCaughey who typed the manuscript.

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