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Altered nerve growth factor in fibroblasts from patients with familial dysautonomia

(cyclic AMP/catecholamines/pro-nerve growth factor/neurologic disease/human genetics)

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ABSTRACT Nerve growth factor was measured in cultured human skin fibroblasts from controls and from patients with familial dysautonomia and dystonia musculoram deformans. Cells from these sources grown over a range of cell densities contained similar levels of β-nerve growth factor as measured by radioimmunoassay. Results of bioassay demonstrated that the nerve growth factor from dysautonomic cells was only approximately 10% as active per ng of immunoreactive protein as that from control and dystonic cells. Treatment of fibroblasts with the β-adrenergic agonist isoproterenol resulted in a 17- to 170-fold rise in the cyclic AMP content of both control and dysautonomic cells in 10 min. The content of immunoreactive β-nerve growth factor in control fibroblasts increased 50–300% after 3–4 hr of exposure to isoproterenol. At no time, throughout a 7.5-hr period, was there a change in the amount of immunoreactive β-nerve growth factor in the dysautonomic cells. These studies suggest that the molecular basis of the genetic defect in familial dysautonomia may lie in processing of the precursor or in the structure of the biologically active β subunit of nerve growth factor.

Familial dysautonomia is an inherited neurologic disease in humans, which primarily affects autonomic and sensory functions (1). Symptoms include postural hypotension, labile body temperature, hyperhydrosis, episodic hypertension, skin blotching, lack of fungiform papillae on tongue, relative insensitivity to pain and temperature, attenuated deep tendon jerks, absence of overflow tears, episodic vomiting, esophageal dysfunction, and lack of histamine flare response (2, 3). This wide array of neuronal dysfunctions correlates with dramatic decreases in neuronal numbers in sympathetic ganglia (4), dorsal root ganglia (5), and parasympathetic ganglia (6). The early onset of this disease (1, 5) and its autosomal mode of inheritance (7) suggest that neuronal development is disrupted when homologous copies of a single gene locus both produce a mutant product.

The molecular nature of the mutant product responsible for familial dysautonomia has not been identified. Nerve growth factor (NGF) has been hypothesized to be the site of the lesion due to the similar pathological alterations in autonomic and sensory development seen in familial dysautonomia and in animals treated with antiserum to NGF (3, 8). By using a radioimmunoassay, Siggers *et al.* (8) found a 3-fold higher level of crossreacting "NGF" in serum from dysautonomic compared with control subjects. By bioassay and binding assay, amounts of NGF appeared similar in these two groups, suggesting increased amounts of a defective NGF in familial dysautonomia. However, there are difficulties in assaying the low levels of NGF in sera because of the presence of NGF-binding components (8, 9).

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The production of NGF by cultured human fibroblasts observed here and by others (ref. 10; I. Schulze and R. Perez-Polo, personal communication) makes it possible to evaluate differences in NGF from patient and control cells grown in a controlled environment, thus avoiding physiologic variables *in vivo*. Skin fibroblasts have proven useful in the diagnosis and study of inherited biochemical lesions in man (11). These cells possess a number of properties important in neuronal function, including activities of enzymes that degrade catecholamines: monoamine oxidase of the A and B types (12, 13), catechol-*O*-methyltransferase (12, 13), and phenolsulfotransferase (14), as well as β-adrenergic and prostaglandin receptors (15, 16). In this study we have used a bioassay and a radioimmunoassay to characterize NGF produced by dysautonomic and control fibroblasts under different conditions of growth and in response to β-adrenergic stimulation. Lines from patients with dystonia musculoram deformans were also studied, because this disease is another inherited peripheral neuropathy with a high frequency in the Ashkenazic Jewish population.

MATERIALS AND METHODS

Cell Culture. Lines of human skin fibroblasts were obtained from the American Type Culture Collection (Rid Mor), from the Institute for Medical Research, Camden, NJ (prefixed with GM), or by outgrowth from biopsy tissues in our laboratory (prefixed with HF). Biopsies from dysautonomic patients were obtained by Felicia Axelrod, and from controls, by Earl Giller. All lines were from Caucasian individuals and were studied prior to senescence. Viable frozen stocks of cells were maintained in the vapor phase of liquid nitrogen (−90°C).

Cells were grown as monolayers on polystyrene tissue culture flasks (75 cm^2) or dishes (150 mm) in the Dulbecco–Vogt modification of Eagle's medium (H-21; GIBCO) supplemented with 10% (vol/vol) fetal calf serum (Flow). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Stock flasks were fed at 5- to 7-day intervals and subcultured 1:5 every 2–3 wk after cell resuspension by treatment with pancreatic hydrolases (pancreatin, GIBCO) in isotonic buffered saline (17). For experiments, cells were plated at a density of 3–6 × 10^5 cells per 150-mm dish. Cell numbers were determined microscopically by using a hemacytometer. A single lot of fetal calf serum was used for all experimental cell growth. Experimental cultures were fed at 3- to 4-day intervals and 1 or 2 days before harvesting.

Harvesting and Preparation of Extracts. Monolayers were rinsed twice with 3 ml of isotonic buffered saline and allowed to drain for 20 sec. Excess fluid was removed and the cells were

Abbreviations: NGF, nerve growth factor; IBMX, 3-isobutyl-1-methylxanthine.

scraped into the residual fluid by using a Teflon-coated straight edge. Cell suspensions were immediately frozen on dry ice and stored at −90°C. To prepare extracts, cell suspensions were homogenized and centrifuged at 1000 × g for 10 min. The supernatant was used as the extract.

Isoproterenol Exposure. L-isoproterenol (Sigma) and 3-isobutyl-1-methylxanthine (IBMX, Aldrich) were solubilized just prior to use. Parallel monolayer cultures were used at 50–90% confluency. Cells were rinsed twice with 3 ml of isotonic buffered saline and then preincubated in 10 ml of medium without serum, with or without 0.5 mM IBMX, for 10 min at 37°C. Isoproterenol (10 μM) was then added and the incubation was continued for the times indicated.

NGF Radioimmunoassay. The β-NGF radioimmunoassay was carried out essentially as described (18, 19) by using antibody raised in rabbits against mouse β-NGF prepared by standard methods (20, 21). Briefly, it involves incubation of antibody against mouse β-NGF with ^{125}I-labeled β-NGF and either β-NGF standards (2.5–40 ng) or cell extracts in 0.1 M Na phosphate buffer, pH 7.4, containing 10 mg of bovine serum albumin per ml for 16–24 hr at 4°C. The antibody–antigen complex was precipitated with protein A (*Staphylococcus aureus*) and its radioactivity was determined in a gamma counter. Standard curves carried out in the presence of cell extracts from control dysautonomic lines were parallel to the control standard curve.

NGF Bioassay. The bioassay was carried out by using dorsal root ganglia dissected from 9-day chicken embryos. Ganglia were incubated for 16–20 hr in 35-mm tissue culture petri dishes containing 1 ml of Ham's F-10 medium supplemented with 20% (vol/vol) heat-inactivated fetal calf serum, 50 units of penicillin per ml, and 50 μg of streptomycin per ml. A standard curve was generated by addition of concentrations of β-NGF over a range of 0.2–5 biological units per ml. Several dilutions of each extract were assayed simultaneously and the biological activity was determined by comparison with the standard curve. Neurite out-growth was rated on a scale from 0 to 4. One biological unit of NGF was defined as that amount which gave a maximal neurite outgrowth response and was produced by 2 ng of purified mouse submaxillary gland β-NGF. The response to cell extracts and purified mouse β-NGF was blocked with antiserum to mouse β-NGF at a final dilution of 1:2000–1:10,000.

Cyclic AMP Assay. Cyclic AMP was measured by a modification of the Gilman binding assay (22). Cells were extracted with 0.3 M HCl; the extracts were lyophilized to remove the HCl and assayed in 50 mM Na acetate, pH 4.

Protein Determination. Protein was determined by the Lowry method (23) with bovine serum albumin as standard.

RESULTS

Cell extracts prepared from human fibroblasts contain "β-NGF" that can be immunoassayed. The immunoassay will detect murine forms of free β-NGF, β-NGF present as part of the 7S NGF complex (at 50–100% efficiency of actual β-NGF content), and, apparently, pro-β-NGF (unpublished results). In this paper immunoreactive β-NGF will refer to all these possible forms. The content of β-NGF, expressed as nanograms of immunoreactive protein, was measured over several stages of growth in cultures of control and familial dysautonomic lines. Levels of β-NGF remained essentially constant or increased slightly as the cells passed from the logarithmic to the stationary phase of growth (Fig. 1). At higher cell densities there were somewhat lower amounts of β-NGF in dysautonomic compared to control cells whether expressed as nanograms of β-NGF per mg of cell protein or per cell number.

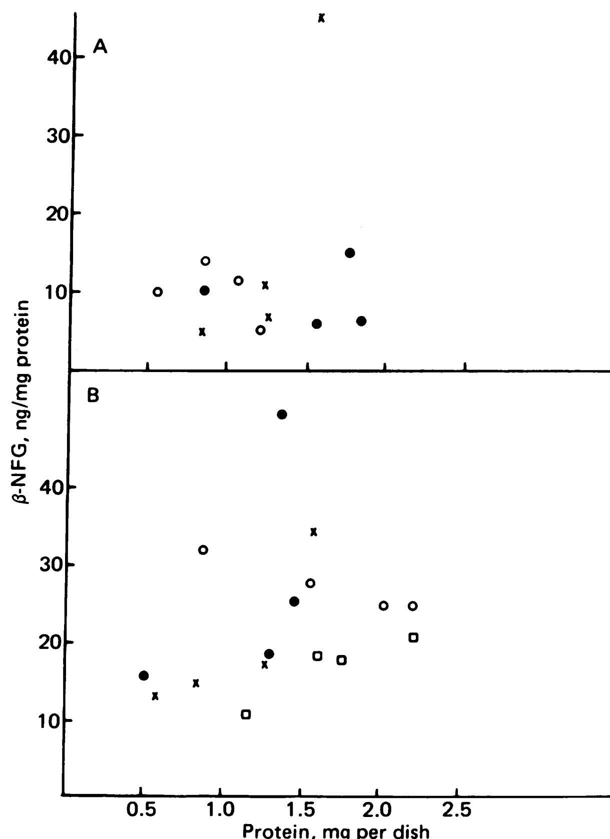

FIG. 1. Immunoreactive β-NGF in control and familial dysautonomic fibroblasts as a function of cell density. Control and dysautonomic cells were seeded in parallel at 3–6 × 10^5 cells per 150-mm dish. Dishes were fed at 2- to 3-day intervals over a 2- to 3-week period. Individual dishes were harvested throughout the growth period at 3- to 5-day intervals beginning when each line reached a level of 50–70% confluency (exceptions are HF9 and HF18, which were first harvested at 90% confluency). Extracts were prepared and β-NGF was assayed by radioimmunoassay. Protein was measured in the same extracts. A duplicate set of dishes was harvested at the same time and cell numbers were determined. Each point represents one dish of the following. (*A*) Familial dysautonomic cell lines: ●, HF55; ○, GM850; x, HF54. (*B*) Control cell lines: ●, HF8; ○, HF9; □, HF18; x, GM23.

Cell extracts were also assessed with respect to their ability to stimulate neurite outgrowth from chicken dorsal root ganglia (Fig. 2). The seven control lines tested had similar levels of β-NGF as assessed by radioimmunoassay and bioassay, ranging from 2 to 6 biological units per ng of immunoreactive β-NGF (Table 1). The high values obtained, compared to pure murine β-NGF, may result from nonspecific effects of other cellular components in the extracts or from the relative efficiencies with which human β-NGF binds to antibodies prepared against mouse β-NGF and to receptors for β-NGF on chicken cells. In contrast to control lines, the five dysautonomic lines tested had a biological activity per ng of immunoreactive β-NGF that was on average 12% that of the controls ($P < 0.001$). For comparison, biological activity and immunoreactive β-NGF was also assessed in extracts of fibroblasts from patients with dystonia musculoram deformans. The dystonic extracts contained levels of biological activity per ng of immunoreactive β-NGF that were similar to control extracts. This suggests some specificity for the alteration in biological activity of the β-NGF from familial dysautonomic fibroblasts. The biological activity of β-NGF measured in extracts from control, dysautonomic, and dystonic fibroblasts was blocked by inclusion of anti-β-NGF

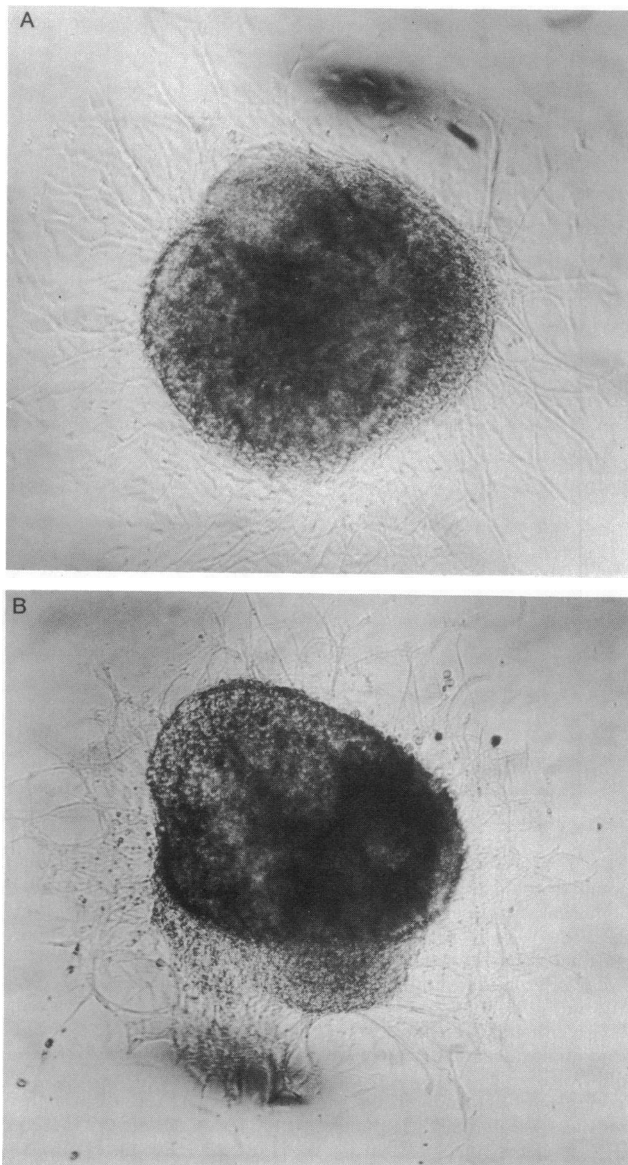

FIG. 2. Stimulation of dorsal root ganglia neurite extension by control and familial dysautonomic fibroblast extracts. The bioassay was carried out as described in *Materials and Methods*. Ganglia are shown after 20 hr of exposure to extracts from: control line HF9 (*A*), with β-NGF at 0.21 ng/ml in the culture (biological activity was determined as 4.8 units/ng of β-NGF) and familial dysautonomic line HF54 (*B*) with β-NGF at 4.3 ng/ml in the culture (biological activity, 0.27 units/ng of NGF). Photographs illustrate 3+ neurite outgrowth.

antiserum in the bioassay. A comparison of parallel cultures of several control and dysautonomic lines over different stages showed no variation within lines of the biological activity per ng of immunoreactive β-NGF. Furthermore, 50:50 mixtures of control and dysautonomic extracts gave additive results in the bioassay [e.g., GM23 + HF55 gave 1.0 unit/ng of β-NGF and HF8 + HF54 gave 1.2 units/ng of β-NGF (see Table 1)].

Recent studies (18, 19, 24) suggest that intracellular cyclic AMP levels may regulate amounts of β-NGF. Human fibroblasts have a β-adrenergic receptor coupled to adenylate cyclase (15, 16). Control and dysautonomic lines exposed to the β-agonist isoproterenol for 10 min in the presence of the phosphodiesterase inhibitor IBMX showed a 17- to 170-fold increase in cyclic AMP levels (Table 2). The magnitude of this increase can be highly variable in fibroblast lines, depending on cell density and days after plating (15, 16), and thus the variations in response between lines are not significant. In control fibroblast lines the presence of isoproterenol resulted in a 50–300% increase in the content of immunoreactive β-NGF measured 3–5 hr later (Fig. 3*A*). In contrast, there was no significant change in the β-NGF content of the dysautonomic cell lines at any point over a period of 7.5 hr after addition of isoproterenol

Table 1. Biological activity of β-NGF in fibroblasts

Cell type		Age, yr	Sex	Biological activity* units/ng (β-NGF)
Control	HF9	22	M	2.0
	HF18	22	F	2.2, 5.2
	HF8	22	M	2.9 ± 0.8 (3)
	GM498	3	M	3.2 ± 0.9 (3)
	GM23	31	F	3.4 ± 0.1 (4)
	86	15	F	4.0, 6.4
	Rid Mor	15	M	6.2
Familial dysautonomia	GM850	26	M	0.33, 1.2
	HF55	19	M	0.37 ± 0.04 (3)
	HF54	24	F	0.46 ± 0.15 (5)
	HF56	17	M	0.46, 0.39
	GM732†	1	M	0.88
Dystonia‡	GM2304	16	F	2.4, 4.0
	GM2255	14	F	3.4, 3.5
	GM2551	31	F	5.3, 3.2

* Extracts of cells were assayed at 3 to 4 dilutions in a bioassay as described in *Materials and Methods*. A radioimmunoassay was carried out on the same extract and the data are presented as the biological units per ng of immunoreactive β-NGF. Values are expressed as mean ± SEM (N) where N = no. of separate extracts used for bioassay. When N = 1 or 2, individual values are given. Most determinations of biologic activity were performed without the rater knowing the type of extract.

† This patient was described as a variant because he did not come from an Ashkenazic Jewish family and had very severe symptoms.

‡ GM2255 and GM2551 are from families manifesting an apparent recessive mode of inheritance; GM2304, from a family manifesting an apparent dominant mode.

Table 2. Effect of isoproterenol exposure on cyclic AMP levels in fibroblasts

Cell line	Isoproterenol*	Cyclic AMP, pmol/mg protein
GM 23	−	48.9 ± 11.3
	+	870 ± 97
HF 8	−	64.4 ± 9.7
	+	1080 ± 150
HF 55	−	37.4 ± 4.2
	+	1600 ± 66
HF 54	−	27.3 ± 6.2
	+	4580 ± 640

* All cultures were incubated with IBMX for 20 min at 37°C and then extracted for cyclic AMP assay. Isoproterenol (10 μM) was added to half the cultures after 10 min of incubation. Values are expressed as in Table 1; N = 3.

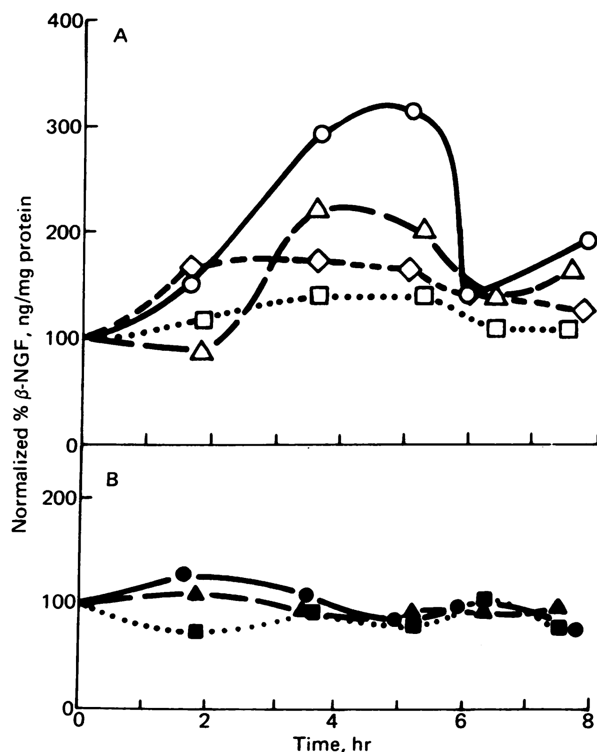

FIG. 3. Time course of effect of isoproterenol on β-NGF content of control and dysautonomic fibroblasts. Cultures were exposed to 10 μM isoproterenol after a 10-min preincubation with IBMX. At the indicated times cells were harvested, and NGF was measured in the extracts of duplicate or triplicate cultures by immunoassay. (Values at time zero are normalized to 100% to allow comparison between lines. Actual levels of immunoreactive β-NGF per mg of protein are given in parentheses.) (*A*) Controls: O, HF9 (12.6 ± 1.3); X, HF18 (9.6 ± 1.1); △, HF8 (27.2 ± 2.3); □, GM23 (44.5 ± 4.1). (*B*) Familial dysautonomics: ●, GM850 (10.1 ± 0.6); ▲, HF55 (27.6 ± 4.3); ■, HF54 (32.7 ± 5.5).

(Fig. 3*B*). The biological activity per ng of immunoreactive β-NGF did not change significantly in control or dysautonomic cells as a result of isoproterenol treatment. Essentially no immunoreactive β-NGF was detected in media from either control or familial dysautonomic cells after 7.5 hr of incubation under these conditions.

DISCUSSION

We propose, based on the data presented in this paper, that the molecular defect in familial dysautonomia is directly related to a defect in the structure or processing of the precursor or in the β subunit of NGF itself. In mice the β subunit is synthesized as a larger precursor polypeptide, which is cleaved by a specific protease, presumably the γ subunit of NGF, into its biologically active form (25). The ability of cultured human fibroblasts to make NGF allows their use in assessing the possible involvement of altered NGF in dysautonomia. When the content of β-NGF in control and dysautonomic fibroblasts was compared by radioimmunoassay, using antibody prepared against mouse β-NGF, the levels were similar. However, when the content of β-NGF was assessed by its ability to promote neurite outgrowth from cultures of embryonic chicken dorsal root ganglia, the activity per ng of immunoassayable NGF in dysautonomic fibroblasts was 10% that of control fibroblasts. NGF from dystonic fibroblasts was similar to controls by these criteria. The cross-species comparison of immunologically and biologically active β-NGF complicates a molecular understanding of these phenomena; still, the parallel comparison between dysautonomia versus control and dystonic extracts is dramatic. Dysautonomic cells appear to produce a variant of β-NGF that crossreacts to antiserum prepared against mouse β-NGF but that is much less biologically active against chicken dorsal root ganglia neurons. Mixing experiments show this cannot be explained by activating or inhibiting factors.

In previous studies (18, 19, 24) one of us (J.P.S.) has observed that the β-NGF content of two brain-derived cell lines, $NB2_A$ neuroblastoma and C6 glioma, varies as a function of intracellular cyclic AMP content. When either cell line is treated with an agonist that raises cyclic AMP levels, there is a corresponding increase in the β-NGF content of the cells 3–4 hr later. The increase in β-NGF occurs by a process independent of new protein synthesis. It has been proposed that a cyclic AMP-dependent protein kinase activates the enzymatic process whereby pro-β-NGF (25) is converted into β-NGF (19). Similar studies were carried out here with human fibroblasts, which contain β-adrenergic receptors coupled to adenylate cyclase (15, 16). Treatment of either the control or the dysautonomic fibroblasts with L-isoproterenol, a potent β-adrenergic receptor agonist, caused a large increase in cellular cyclic AMP levels within 10 min. The immunoreactive β-NGF content of the control cell lines increased (50–300%), with a maximal response occurring 4–5 hr after addition of isoproterenol, whereas the β-NGF content of the dysautonomic lines did not change during 7.5 hr of exposure. This finding suggests a possible defect in the processing of pro-β-NGF into β-NGF in familial dysautonomia. The defect could result from an alteration in the enzyme responsible for processing of this precursor molecule, in a cyclic AMP-dependent protein kinase that may activate processing, or in the structure of the precursor molecule for β-NGF. Any of these mechanisms could result in the presence of an immunologically recognized protein that is biologically less active and is converted with decreased efficiency to the biologically active form after a rise in cyclic AMP. A structural alteration in the precursor form of β-NGF might be retained in the processed form and compromise its biological activity. The present studies show a clear difference in biologic and immunologic properties of β-NGF from control and dysautonomic fibroblasts. Understanding of the molecular mechanisms underlying this difference can only be inferred and awaits further biochemical analysis.

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