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Determination of Nerve Growth Factor Concentrations in Human Samples by Two-Site Immunoenzymometric Assay and Bioassay

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Summary: Nerve growth factor is a neurotrophic protein which is known to act on sympathetic and sensory neurons and on the magnocellular cholinergic neurons of the basal forebrain. We quantified nerve growth factor in human tissues and body fluids by two methods, a rapid and sensitive two-site immunoenzymometric assay and a bioassay using dissociated chick dorsal root ganglion neurons. The two-site immunoenzymometric assay detects nerve growth factor in concentrations as low as 0.5–2.5 ng/l. Using a monoclonal antibody to mouse nerve growth factor, we found that the signal of the antibody for recombinant human nerve growth factor is about 60–90% of the signal for mouse nerve growth factor. As a control for the specificity of our data, a bioassay for nerve growth factor was performed and the results showed a good correlation. The highest nerve growth factor concentrations were found in sciatic nerve (2.5 ng/g wet weight), cardiac atrium muscle (1.5 ng/g wet weight) and in the central nervous system in the hippocampus (1.9 ng/g wet weight). Lower nerve growth factor concentrations were measured in human sera (0.2 ng/g wet weight). No nerve growth factor was detectable in cerebrospinal fluid. The distribution of human nerve growth factor-rich tissues is similar to that reported for rat tissues.

Introduction

Nerve growth factor is a neurotrophic protein which is synthesized by target tissues of nerve growth factor-sensitive neurons, selectively taken up by the nerve terminals and transported retrogradely to the cell bodies. Nerve growth factor has been shown to act on sympathetic and neural crest-derived sensory neurons in the peripheral nervous system as well as on the magnocellular cholinergic neurons of the basal forebrain (for review, see l.c. (1–3)). It has been suggested that nerve growth factor is involved in the neuropathology of *Alzheimer*'s disease (4). In the peripheral nervous system nerve growth factor may play a role in the development of autonomic and sensory neuropathies (5). To clarify whether nerve growth factor is of physiological or pathological significance in humans the sensitive and reliable quantification of endogenous nerve growth factor in human tissues and body fluids is a prerequisite.

The genomic sequence of human nerve growth factor has been determined (6) and small quantities of biologically active recombinant human nerve growth factor have already been produced (Genentech, USA). Since the sequence homology between murine and mature human nerve growth factor is 86% on the nucleotide level and 90% on the amino acid level (6), a relatively high immunological cross-reactivity is to be expected. Since the levels of nerve growth factor in tissues are extremely low in general, there is a need for a highly sensitive test system. The bioassay for nerve growth factor, which is based on the neurotrophic effect of nerve growth factor on embryonic sensory neurons (7), requires time-consuming cell culture procedures and is not suitable for measurements in a large number of samples. In competitive immunoradiometric assays, problems arise from the unspecific binding of nerve growth factor, for example by α_2-macroglobulin (8, 9). A two-site immunoradiome-

tric assay (8) yielded reliable results, but did not attain the sensitivity required for measurements in human tissues and body fluids. Two-site immunoenzymometric assays (10, 11) allowed a reliable and sensitive determination of nerve growth factor concentrations, but required sophisticated equipment for coating glass beads or polystyrene tubes with antibody and for fluorometric measurement of the substrate reaction. Based on the fluorometric methods described (10, 11), we therefore developed an assay (12, 13) which uses a chromogenic substrate and which is adapted to microtitre plates. We employed this method to determine nerve growth factor concentrations in human tissues, cerebrospinal fluid and serum using recombinant human nerve growth factor as a standard. Additionally, we examined the specificity of our data by determining the nerve growth factor concentrations in a sensory neuron bioassay.

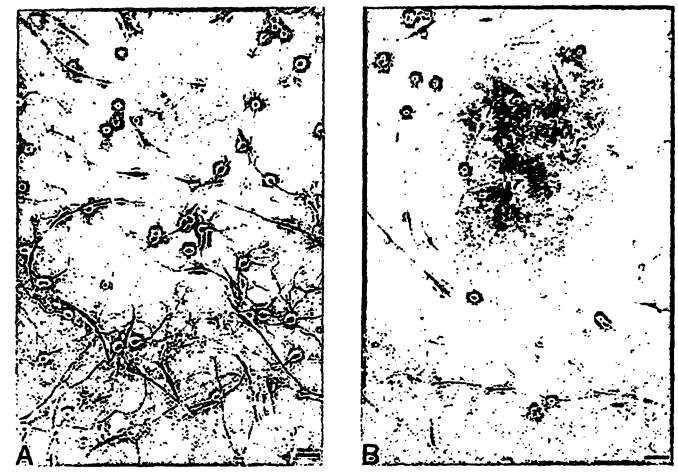

Fig. 1. Bioassay for nerve growth factor: Phase (-contrast) micrographs of dorsal root ganglia neurons cultured for 48 h in the presence of recombinant human nerve growth factor:
(a) 500 ng/l medium; (b) 5 ng/l medium
calibration bar = 50 µm.

Materials and Methods

Two-site immunoenzymometric assay

The immunoenzymometric assay was performed as a two-site assay using the same antibody on both sites, since nerve growth factor is a homo-dimer consisting of two identical subunits. We employed the anti mouse nerve growth factor monoclonal antibody MAB 27/21 (10). This antibody, as well as our protocol for the assay, is now available from Boehringer Mannheim, Germany. Rigid microtitre plates with high binding capacity and low variation coefficient (Immuno, Nunc) were coated with antibody protein overnight at 4 °C (0.3 mg protein per litre carbonate/bicarbonate buffer 0.05 mol/l; pH 9.7). The plates were washed with Tris buffer (0.05 mol/l Tris; 0.15 mol/l NaCl; 0.005 mol/l $MgCl_2$; 1 g/l Triton-X-100; pH 7.0) and incubated at 4 °C overnight with standard solutions of recombinant human nerve growth factor (2 samples of recombinant human nerve growth factor, lot 1 and lot 2 were obtained from Genentech, USA), mouse nerve growth factor (Boehringer Mannheim, Germany) or samples, diluted in Tris buffer supplemented with bovine serum albumin (10 g/l). The concentration of mouse nerve growth factor in the standard solutions was monitored by determination of absorbances and by determination of the half-maximal survival in the bioassay (see below). The microtitre plates were washed again before the antibody coupled to β-galactosidase[1]) (Boehringer Mannheim) was added (400 U/l buffer, containing 0.05 mol/l Tris, 0.15 mol/l NaCl, 0.005 mol/l $MgCl_2$, 1 g/l Triton-X-100, 10 g/l bovine serum albumin; pH 7.00) and again incubated overnight at 4 °C. The indicator reaction used chlorophenol red-β-galactopyranoside (Boehringer Mannheim) as a chromogenic substrate. After an incubation period of at least 1 hour, the absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 630 nm. We determined a standard curve in triplicate on each microtitre plate.

Bioassay

The in vitro bioassay (fig. 1) is based on the fact that neural crest-derived embryonic sensory neurons survive and extend neurites in culture in the presence of nerve growth factor, whereas they die rapidly in its absence (7, 14–16). Chick dorsal root ganglia (embryonic day 8) were prepared and collected in phosphate-buffered saline. The ganglia were incubated in phosphate-buffered saline containing 1 g/l trypsin (Worthington) and dissociated by gentle trituration with a siliconized *Pasteur* pipette. The cells were then suspended in F14 medium (Gibco) supplemented with horse serum (100 ml/l medium; Gibco), preplated in 100 mm dishes (Nunc) in order to remove non-neuronal cells, then plated into 24-well culture dishes (Costar) coated with polyornithine (0.5 g/l in borate buffer; Sigma) and laminin (4 mg/l; a gift from *D. Edgar*). Samples, or standard dilutions of mouse nerve growth factor and recombinant human nerve growth factor in the range of 5–2000 ng/l medium were added. The rate of surviving neurons was dose-dependent at concentrations of nerve growth factor ranging from 20–500 ng/l culture medium, mouse nerve growth factor showing equivalent activity to that of recombinant human nerve growth factor. The biological activity of both mouse nerve growth factor and recombinant human nerve growth factor was blocked by the specific anti-mouse nerve growth factor monoclonal antibody (MAB 27/21). For all measurements, the addition of MAB 27/21 (in final concentrations of 10–200 µg/l culture medium) to parallel samples served as a control to distinguish nerve growth factor-specific survival of the cells from effects of other uncharacterized factors. The detection limit of the nerve growth factor bioassay is in the range of 10–20 ng/l cell culture medium.

Preparation of tissue samples

Human tissues were obtained from the Department of Forensic Medicine, University of Munich 4.5–20 h post mortem. Adult Wistar rats were killed by cervical dislocation. Bovine tissues were obtained from the municipal slaughterhouse 0.5 and 24 h post mortem. Tissues were frozen in liquid nitrogen and stored at −80 °C. After homogenization at 4 °C in Tris buffer (0.1 mol/l Tris, 0.3 mol/l NaCl, 20 g/l bovine serum albumin; pH 7.0), containing aprotinin (20 000 kallikrein units per litre), 0.1 mmol/l benzethoniumchloride (Serva), and 1 mmol/l benzamidine, the homogenates were ultracentrifuged. For the EIA, the supernatants were diluted 1 + 1 with buffer (containing 0.01 mol/l $MgCl_2$ and 2 g/l Triton-X-100; pH 7.0). For the biosassay, the supernatants were diluted with F14 medium containing horse serum (100 ml/l medium; Gibco) and filtered using 2 µm filters (Amicon). Unselected sera and cerebrospinal fluid samples were freshly obtained continuously from our diagnostic

[1]) Enzyme β-galactosidase (β-*D*-galactoside galactohydrolase EC 3.2.1.23)

laboratory. For the bioassay, sera were incubated for 30 minutes at 51 °C to remove cytotoxic complement activity. Final dilutions of samples ranged between 1 : 10 and 1 : 20. For all samples the recovery of nerve growth factor was determined by adding 0.05–0.5 pg/l recombinant human nerve growth factor to one part of the homogenate before the homogenates were ultracentrifuged and by calculating the fraction of recovered nerve growth factor. False positive results in a two-site assay might be found when anti-mouse IgG present in a sample „crosslinks" the first and second monoclonal antibody. We ruled out this possibility by incubating samples and standards with unspecific monoclonal antibodies (Blockierungsreagenz CK 33, available on request from Boehringer Mannheim) in different concentrations from 0.6–42 mg/l in order to bind anti-mouse IgG.

Results

An immunoenzymometric assay for nerve growth factor based on a chromogenic detection system using β-galactosidase and chlorophenol red-β-galacotopyranoside was developed. In order to adapt the assay to microtitre plates, different types of plates including vinyl, polystyrene and activated polystyrene plates were compared, and activated polystyrene plates were selected for highest binding capacity. Coating concentrations for the nerve growth factor antibody and nerve growth factor antibody-β-galcatosidase conjugate were optimized. For blocking the plates after antibody adsorption, buffers containing bovine serum albumin, horse serum, rat serum and ovalbumin were compared, and it was found that bovine serum albumin in a concentration of 10 g/l yielded the best blocking results. For the incubation step with nerve growth factor, different buffer, pH and detergent conditions were tested; it was found that coating at pH 9.7 and incubation at pH 7.0 using the detergent Triton-X-100 gave the best results.

Furthermore, all incubation steps were performed with various combinations of the following incubation conditions: incubation at 4 °C overnight, at room temperature for two hours, or at 37 °C for two hours. All of these variations yielded standard curves, but for all incubation steps, incubation at 4 °C overnight yielded the lowest and incubation at 37 °C for two hours yielded the highest standard deviations for the triplicate measurements. We therefore decided to perform all measurements with human nerve growth factor by incubating at 4 °C overnight, since in human tissues relatively low nerve growth factor concentrations near the detection limit of the assay were expected. Nevertheless, it is possible to perform the assay by incubating for two hours at room temperature and thus achieve good results in the medium range of sensitivity within.

Under these optimized assay conditions the detection limit of the nerve growth factor assay was 0.5 ng/l (the detection limit was defined as three standard deviations of the blank signal). Figure 2 shows a typical standard curve. When the more rapid assay protocol with incubation at room temperature was used, the detection limit was 2.5 ng/l. The intra-assay variance of the optimized assay was determined by assaying a sample 18 times on one microtitre plate. The inter-assay variance was estimated by analysis of 4 different samples in 18 consecutive runs. The reproducibility is given in table 1. To examine the specificity of the assay, insulin and lysozyme were tested as antigens. Neither of these gave a signal. When comparing mouse nerve growth factor and recombinant human nerve growth factor, the signal for recombinant human nerve growth factor was about 60% of the signal for the same concentration of mouse nerve growth factor in lot 1 of human nerve growth factor and 90% of the signal of mouse nerve growth factor in lot 2 of human nerve growth factor. To find out

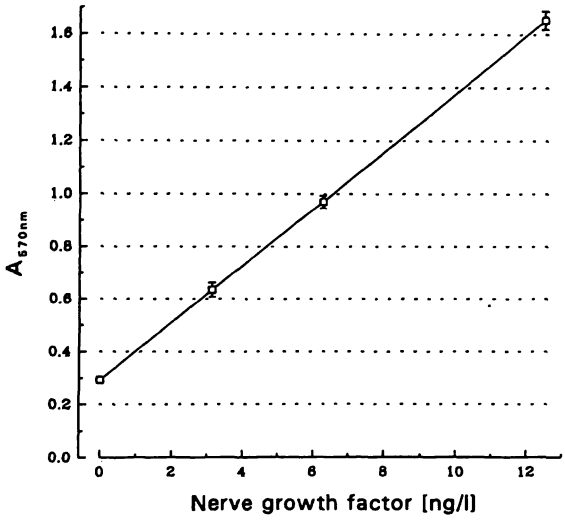

Fig. 2. Typical standard curve of the enzyme immunoassay for nerve growth factor for measurements in the lower range of sensitivity.

Tab. 1. Nerve growth factor assay precision

	n	Nerve growth factor measured (ng/l) mean ± standard deviation	Coefficient of variation (%)
Intra-assay precision			
Sample 40 ng/l	18	41.43 ± 1.31	3.15
Inter-assay precision			
Sample 6.25 ng/l	18	6.41 ± 0.48	7.50
Sample 50 ng/l	18	49.94 ± 1.67	3.33
Sample 100 ng/l	18	100.06 ± 1.52	0.99
Sample 200 ng/l	18	200.32 ± 0.54	0.27

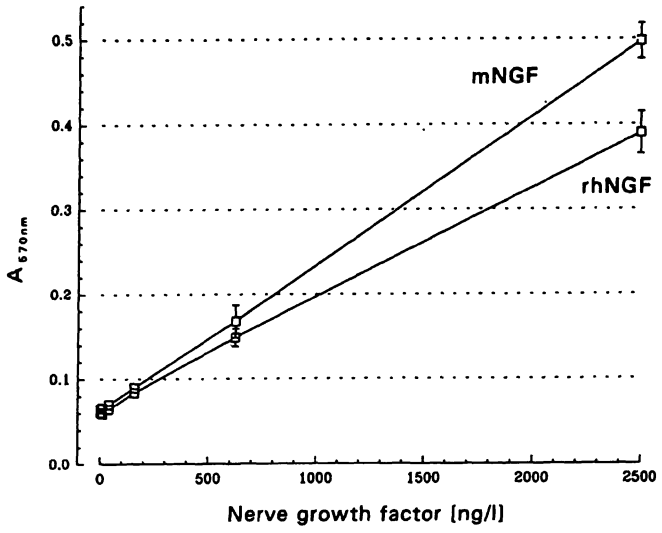

Fig. 3. Comparison of standard curves for mouse nerve growth factor (mNGF) and recombinant human nerve growth factor (rhNGF) from lot 2. The data for the two graphs were determined on the same microtitre plate.

whether this difference is due to different concentrations of recombinant human nerve growth factor in the two samples received, we tried to further evaluate the nerve growth factor concentration in the samples by bioassay and measurement of optical density, but the amount of recombinant human nerve growth factor we could obtain from the two lots was so small that these experiments were not possible. Figure 3 shows a comparison of the standard curves of mouse nerve growth factor and recombinant human nerve growth factor from lot 1.

For testing samples in the bioassay it is necessary to remove cytotoxic complement activity by incubating the samples for 30 minutes at 51 °C. In order to find out whether this incubation leads to a degradation of nerve growth factor we assayed samples before and after incubation in the immunoenzymometric assay. There was a decrease of 14% in the nerve growth factor concentration in tissue homogenates and of 22% in serum samples. This decrease was compensated for in each sample by the calculation of the recovery of nerve growth factor, which was exogenously added before the incubation step (as described in the section, preparation of tissue samples). In order to quantify a possible decrease of nerve growth factor in post mortem tissues, we determined the nerve growth factor concentrations in rat heart, sciatic nerve, hippocampus and the rest of the brain immediately post mortem and 6 and 24 hours post mortem and in bovine hippocampus, temporal cortex, occipital cortex and spinal cord 0.5 and 24 hours post mortem. When preparing the tissue samples, it was noted that rat brain tissues 6 and 24 hours post mortem already showed macroscopic colliquative changes, whereas rat heart, bovine tissues and human post mortem tissues appeared macroscopically intact. In bovine tissues and rat heart tissues no relevant decrease in nerve growth factor content was found. The mean nerve growth factor levels in rat nervous tissues 6 hours post mortem were 21% lower than immediately post mortem; 24 hours post mortem nerve growth factor levels had decreased by 37%. Nerve growth factor determinations in rat heart 0, 6 and 24 hours post mortem, bovine brain tissues 0.5 and 24 hours post mortem and human tissues 4.5 and 20 hours post mortem showed no relevant differences in nerve growth factor content related to the period post mortem.

Nerve growth factor concentrations were determined in various human post mortem tissues. The recovery of exogenously added recombinant human nerve growth factor was in the range of 70 to 100%. Nerve growth factor measurements in human heart atrium muscle, skeletal muscle, sciatic nerve, temporal cortex, hippocampus, and cerebellum were performed by the immunoenzymometric assay and the bioassay in parallel. Relatively high nerve growth factor concentrations were found in sciatic nerve and cardiac atrium muscle. In the central nervous system, the highest nerve growth factor concentrations were measured in the hippocampus (tab. 2).

When parallel measurements using immunoenzymometric assay and bioassay were performed in human sera and cerebrospinal fluid samples, the recovery of exogenously added recombinant human nerve growth

Tab. 2. Nerve growth factor concentrations in human tissues and body fluids

	Immuno-enzymometric assay nerve growth factor ng/l wet weight	Bioassay nerve growth factor ng/g wet weight
Cardiac atrium muscle	1.5 ± 0.4	0.8 ± 0.5
Skeletal muscle	0.3 ± 0.01	not detectable
Sciatic nerve	2.4 ± 0.5	2.6 ± 0.5
Temporal cortex	1.2 ± 0.02	1.5 ± 0.5
Hippocampus	1.9 ± 0.3	2.0 ± 0.5
Cerebellum	0.9 ± 0.2	0.5 ± 0.5
Cerebrospinal fluid	not detectable (n = 92)	not detectable (n = 5)
Serum	0.2 ± 0.02 (n = 30)	0.17 ± 0.01 (n = 29)

Values given are means ± standard deviation. For tissues, five independent experiments (involving samples from 5 different individuals) were performed, each involving triplicate determinations. For body fluids, the number of independent experiments (n) is given in parenthesis; each experiment was performed in triplicate.

factor was 50 to 110%. Nerve growth factor concentrations were in the range of 200 ng/l in serum. No nerve growth factor was detectable in cerebrospinal fluid (tab. 2). Since the body fluids were diluted by a factor of 1 : 10 for the assays, the limit of nerve growth factor detection was about 20 ng/l sample in the EIA and 150 ng/l sample in the bioassay.

Discussion

We optimized an immunoenzymometric assay to permit the detection of nerve growth factor in concentrations as low as 0.5 to 2.5 ng/l. The assay can be carried out in microtitre plates and this permits rapid handling of a large number of samples. Since the indicator reaction uses a chromogenic substrate, it can be followed photometrically and there is no need for sophisticated equipment. The signal for recombinant human nerve growth factor in the assay varied in the range of 60 to 90% of the signal for mouse nerve growth factor in different lots of recombinant human nerve growth factor. This affinity and sensitivity should be sufficient for sensitive quantification of human nerve growth factor, since the nerve growth factor concentrations reported, for example in rat tissues, are in the range of 0.1 – 1.0 ng/g wet weight (10, 17). To examine the specificity, insulin, which shows partial sequence homologies with nerve growth factor (6), and lysozyme, which is a strongly basic protein like nerve growth factor, were tested and gave no signal in the assay. When we measured nerve growth factor in rat, bovine and human tissues at different times in the first 24 hours post mortem, we found no relevant decrease in nerve growth factor content, except in rat brain tissues, where the decrease is probably due to the pronounced colliquative changes.

We quantified nerve growth factor in human tissues and body fluids by two different methods; the immunoenzymometric assay and a nerve growth factor bioassay using dissociated chick sensory neurons. The nerve growth factor bioassay serves as a control for the specificity of the EIA, since the signal depends on the binding of nerve growth factor to its receptor on cells in culture; the receptor binding site of the nerve growth factor molecule has been highly conserved during the evolution of vertebrates (7). We found comparable results for the immunoenzymometric assay and bioassay measurements.

The highest nerve growth factor concentrations were found in sciatic nerve, which contains sensory fibres, and in heart atrium muscle, which is sympathetically innervated. In the central nervous system the highest nerve growth factor levels were measured in hippocampus and cortex, which are target tissues of the magnocellular cholinergic system of the basal forebrain. This means that the nerve growth factor concentration correlates with the physiological distribution of nerve growth factor-sensitive neurons and their target tissues. The observed distribution of nerve growth factor-rich tissues is similar to that reported for rat tissues (10, 17), which was also confirmed by our immunoenzymometric assay.

In comparison with nerve growth factor levels in tissues containing nerve growth factor-sensitive neurons, relatively low nerve growth factor concentrations in the range of 200 ng/l were measured in human sera, while the signal was not detectable in cerebrospinal fluid samples. In early studies using one-site immunoradiometric assay, much higher nerve growth factor levels were detected in mouse sera, but these results could not be confirmed by bioassay or two-site immunoradiometric assay (8). Bioassay and two-site immunoradiometric assay measurements are limited by the sensitivity of these assays, but they showed that the nerve growth factor concentration was lower than 5000 ng/l by the two-site immunoradiometric assay and lower than 2000 ng/l serum by the dorsal root ganglion bioassay (8). *Beck* et al. (18), using a more sensitive two-site immunoradiometric assay, found that the nerve growth factor levels in human serum are lower than 500 ng/l. *Stephani* et al. (19) evaluated serum nerve growth factor levels with a sensory neuron bioassay, and reported concentrations of 200 to 1000 ng/l, in the same range as our results. These low but measurable nerve growth factor levels in human body fluids indicate that the primary function of nerve growth factor is a local trophic effect, but do not exclude an additional systemic effect.

To determine whether nerve growth factor has a pathological or physiological significance in humans, nerve growth factor concentrations can now be determined in a relatively large number of human sera, cerebrospinal fluid samples and post mortem tissues and correlated with the clinical data. Monoclonal antibodies against human nerve growth factor should be developed as soon as suitable quantities of recombinant human nerve growth factor are available.

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