Effects of Exogenous NGF on Cell Infiltration and Level of Heat Shock Protein-27 in the Central Nervous System

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Abstract

Nerve growth factor (NGF) is the first characterized member of the neurotrophin family. It is known for its crucial role in survival, differentiation and maintenance of neurons both in peripheral and central nervous system. In addition to its neurotrophic role, NGF has been also proposed to influence the immune system. Recent studies indicate that in multiple sclerosis and in its animal model (EAE) there is an increased level of NGF in acute phase of the disease (relapses) and conversely decreased level during the remission phase. Increased level of NGF has been also reported in other autoimmune diseases such as lupus erythmatosus. Concomitantly, proinflammatory cytokines are upregulated in the acute phase of autoimmune diseases, known to be as potent inducers of heat shock protein expression. These observations suggest that over production of NGF in CNS may functionally be related to the state of activation of the immune system in autoimmune diseases. In this study, we have investigated whether increased level of NGF in CNS triggers the immune system. NGF was therefore injected intracerebroventricularly at doses 5 and 20 μg/mice for four days. Our histological and immunohistochemical results show that there are no signs of immune cell infiltration and no changes in the level of heat shock protein expression in different areas of the brain. We therefore suggest that either NGF alone is unable to trigger the immune system or the NGF regimen used in this study was insufficient to do so.

Introduction

Nerve growth factor (NGF), the first identified neurotrophin, has various effects on growth, proliferation and differentiation of neural crest derived and sympathetic neurons (Bueker *et al*., 1948; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Angelletti, 1963; Levi-Montalcini 1987; Aloe *et al.,* 1997). NGF also interferes with the immune system and it is therefore conceivable that any disturbances in its activity or synthesis may interrupt functions in both nervous and immune systems (Levi-Montalcini *et al*., 1990; D'Souza *et al.,* 1994; Levi-Montalcini *et al*., 1996; Aloe *et al.,* 1997).

Otten and colleagues (1995) have reported an increased level of NGF in cerebrospinal fluid of multiple sclerosis patients during disease attacks (relapses) and conversely a reduction during the remitting phase (disease silence). Moreover, NGF level is increased in EAE animal model of multiple sclerosis and in autoimmune diseases such as lupus erythmatosus and arthritis rheumatitis (Aloe *et al*., 1992; Bracci-Laudiero *et al*., 1992, 1993; Otten *et al*., 1995). This increase in NGF in autoimmune diseases suggests a presumptive role of NGF in their incidence.

In vitro and *in vivo* experiments have indicated NGF as a chemotactic factor for polymorphonuclear cells (Boyle *et al*., 1985). Due to concomitant incidence of leukocyte infiltration in CNS and increased level of NGF in inflammatory sites and around the blood vessels in acute phase of an autoimmune disease like multiple sclerosis, one may question if NGF has any effect on immune cell infiltration.

Evidence have also indicated that there is an increased level of inflammatory cytokines in autoimmune diseases (Thoenen *et al*., 1987; Otten and Gadient, 1995; Micera *et al*., 1995; Scolding, 1999) which in turn will affect on other factors such as heat shock proteins (D'Souza *et al*., 1994). Heat shock proteins also act as potential autoantigens and trigger, amplify or modify the autoimmune responses (Gonzalez *et al*., 1989; Aquino *et al*., 1997). Increased expression of HSP in rheumatoid joints in arthritis and in   cells of pancreas in diabetes, the existence of HSP reactive T cells in autoimmune diseases (Cohen, 1991; Yang and Feige, 1991; Brudzinsky *et al*., 1992; Anderson *et al.,* 1995) are some supportive examples for the HSP role

in triggering the immune response. Therefore, to indirectly address the possible effect of NGF on the synthesis of cytokines and triggering the immune response, changes in the level of heat shock proteins (HSP) could be investigated.

In the present study, we therefore first purified NGF followed by testing its biological activity both *in vivo* and *in vitro (*bioassay). Making sure of its biological activity, we then applied it intracerebroventricularly to see if there is any effect on the level of HSP and immune cell infiltration.

Materials and Methods
Animals
For NGF purification, 200-250 adult male mice (Razi institute) were used. For *in vivo* and *in vitro* assays of biological activity, neonatal rat pups and fertilized eggs (Sadra Company) were used respectively. For NGF infusions, 6-8 week old SJL/J female mice were purchased (Charles River) and after a week or two acclimatization period used.

Treatments
To perform an *in vivo* assay for biological activity of NGF, it was administered intraperitoneally at a dose of 0.3 mg/kg in newborn rat pups every other day for one week. Control group received PBS. On day 8, superior cervical ganglia removed, cleaned of the surrounding tissues and weighed.

To perform an *in vitro* bioassay for biological activity of NGF, dorsal root ganglia from chick embryos (embryonic day 8) were dissected (using a stereomicroscope) and cultured in DMEM culture media containing 20% collagen. NGF at concentrations ranging from 50-200 ng/ml was used. Control ganglia did not receive NGF.

To inject NGF intracerebroventricularly, a canule was placed in the right ventricle stereotactically (the coordinates were 0.6 mm posterior, 1.5 mm lateral and 3.5 mm vertical with reference to bregma; according to Franklin and Paxinos, 2003). Following a week of recovery, NGF was injected at doses of 5 and 20 μg/mice. Control group received a solution of PBS (phosphate buffered saline) instead.
?₁*NGF purification*

NGF was purified from mouse salivary glands according to the method of Mobley and colleagues (1976). Briefly, 200 adult male mice, provided 20-30 g of glands, were dissected out and the surrounding fine membrane removed, either homogenized immediately after and processed or kept frozen at -70$^{?}$c until use. The homogenized glands were centrifuged thereafter (35,000 rpm, 1hr, 4$^{?}$c), supernatant taken off and dialysed against 20mM phosphate buffer, PH 6.8, 4$^{?}$c for 16 hr. The dialysed supernatant was then passed through a CM sepharose fast flow column (equilibrated with 20 mM phosphate buffer PH 6.8) and washed with the same buffer at a flow rate of 50 ml/hr, using an electric pump, until the 280 nm O.D. was < 0.5. The whole eluate collected and dialysed against 3x4l of 0.25mM phosphate buffer , PH 6.8, 4$^{?}$c for 24 hr. To the dialysed eluate, 0.5M sodium buffer, PH 4 was added (1/9 volume of the eluate) to reach a concentration of 0.05M, followed by adding 4M NaCl (1/9 volume of the eluate). This will result in a precipitant which should be separated by centrifugation. The supernatant is then loaded on a second column of CM sepharose equilibrated with 0.05M sodium acetate buffer in 0.4M NaCl (With a flow rate of 50ml/hr). After an extensive wash with the same buffer (O.D~0) to get rid off the non adsorbed material, an overnight wash with 0.05M sodium acetate buffer in 0.4M NaCl is required. Following a wash with 0.05M Tris/HCl buffer, PH 9.0, NGF can be eluted with a 0.05M Tris/HCl buffer in 0.4M NaCl, PH 9.0. All fractions should be collected by using a fraction collector, pooling those with the O.D>0.05. The fractions could be concentrated down to 1 mg/ml using an amicon positive pressure filtration. The concentrated fraction can then be dialysed against 10mM sodium acetate buffer PH 5.0. The dialysed material could then be aliquoted and frozen in -70$^{?}$c until use. Using SDS-PAGE (polyacrylamide gel electrophoresis), the purified NGF was electrophoresed followed by *in vitro* and *in vivo* assays for its biological activity.

Histology and immunohistochemistry

Brains were placed in Bouin's fixative, paraffin blocked, sectioned and stained using H&E staining. Sections were then investigated for signs of cell infiltration. Also paraffin slides were dewaxed and processed for immunohistochemistry. Briefly, slides were incubated in H2O2 (3%, 10 min), washed with PBS-Triton X-100 (5 times, 3 minutes), blocked with blocking buffer (5% normal rabbit serum+0.8% BSA+ 0.25% Triton X-100, 30min, 37$^{?}$C). They were then incubated in primary antibody (Goat anti-IgG HSP-27, 1/100, 2hr, 37$^{?}$C; Santa Cruz), washed in PBS-Triton X-100 (5 times, 3 minutes) followed by an incubation in secondary antibody (rabbit biotinylated anti goat IgG, 1/250, 2 hr, RT; Sigma). Slides were then washed in PBS-Triton X-100 (5 times, 3 minutes) and incubated in ABC solution (Avidin-biotin-horseradish peroxidase, 1/100, 1 hr; DAKO). Finally, after washes in PBS-Triton X-100 (5 times, 3 minutes), the reaction was developed using DAB (Diaminobenzidine tetrahydrochloride, DAKO, 2 min). Development was terminated by washes with PBS (5 times, 3 minutes) and counterstaining was performed by using haematoxilin. Slides were then dehydrated and mounted.

Results

Using SDS-PAGE, the purified NGF was tested for its physical properties (e.g. molecular weight). NGF is a dimer of 26 kDa which is converted to a monomer of 13 kDa after being boiled prior to electrophoresis. Our results in figure 1 show that the extracted NGF is pure with a single band of 13 kDa. Applying our purified NGF *in vivo,* we were able to show that it is biologically active and able to increase size and weight of the SCGs in newborn pups (Fig. 2). Also our results from *in vitro* bioassays show that NGF is able to induce neurite outgrowth from the dorsal root ganglia of chick embryos at doses 50-200 ng/ml (Fig. 3a, b) in comparison with control ganglia receiving no NGF (Fig. 3d). As indicated elsewhere a concentration of 200 ng/ml
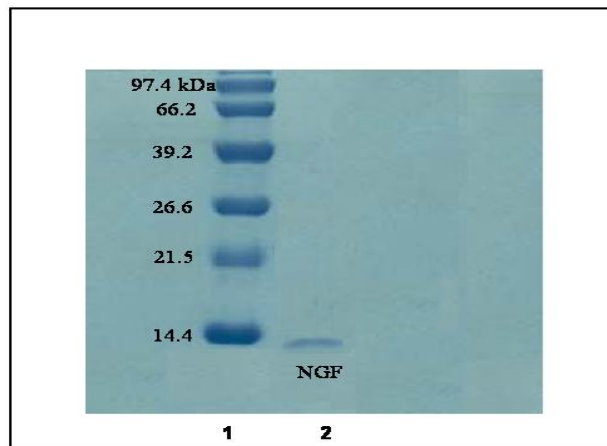
Figure 1- Electrophoresis of the extracted NGF. Protein marker (lane 1), a monomer of 13 kDa NGF (lane 2).
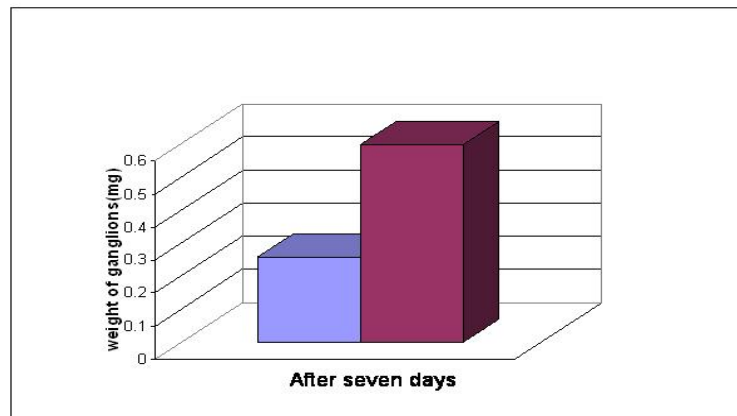

Figure 2- An *in vivo* assay for biological activity of the extracted NGF. Weight analysis of the SCGs taken from new born rat pups treated either with 0.3 mg/kg of the extracted NGF or PBS show that there is a significant increase after the NGF treatment ($p < 0.001$).
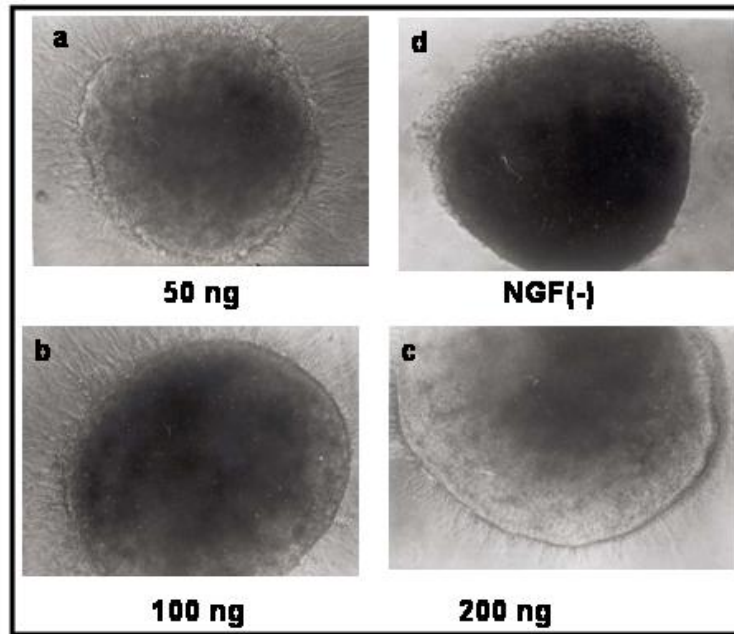
Figure 3- An *in vitro* assay for biological activity of the extracted NGF. NGF is able to induce neurite outgrowth from the dorsal root ganglia of chick embryos at doses 50-200 ng/ml (a, b and c) in comparison with control ganglia receiving no NGF (d).

(Fig. 3c) is not as potent as 100 ng/ml in inducing neurite outgrowth and therefore the optimum concentration would be 100 ng/ml.

Making sure that our extracted NGF is biologically active, we then applied it in intracerebroventricularly at doses of 5 and 20μg/mice. Comparative histological investigation of brain in control and NGF treated animals (Fig.4) show that NGF treatment has not induced perivascular leukocyte infiltration (5, 20 μg/mice; Figs. 4b, 4d). Also immunohistochemical analysis of HSP immunoreactivity (HSP-ir) in brain stem did not show any significant changes in the intensity of the HSP-ir following NGF treatment (Fig. 5b). Moreover, in other areas of the brain such as striatum with low or no expression of HSP, no induction of HSP immunoreactivity can be seen after NGF treatment (Fig. 5d). Altogether, these results indicate that NGF treatment neither has affected on cell infiltration nor on the HSP level in brain.
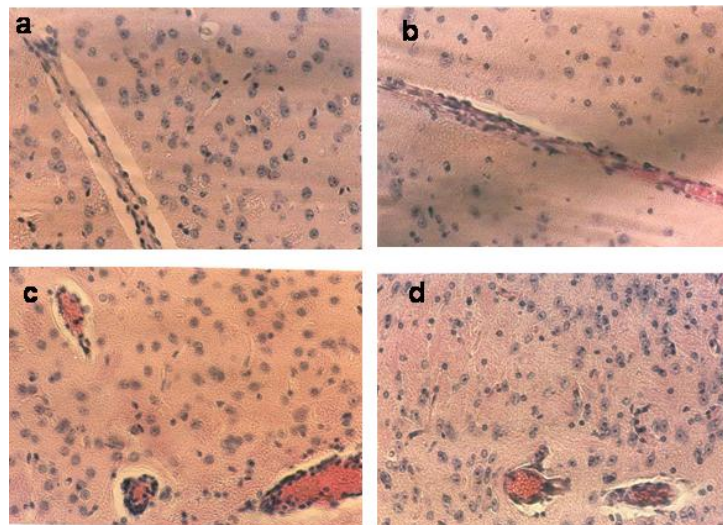
Figure 4- Photomicrographs of H&E staining of paraffin sections from brains of the animals treated with NGF (b: 5μg/mice, d: 20μg/mice) and untreated animals (a, c).
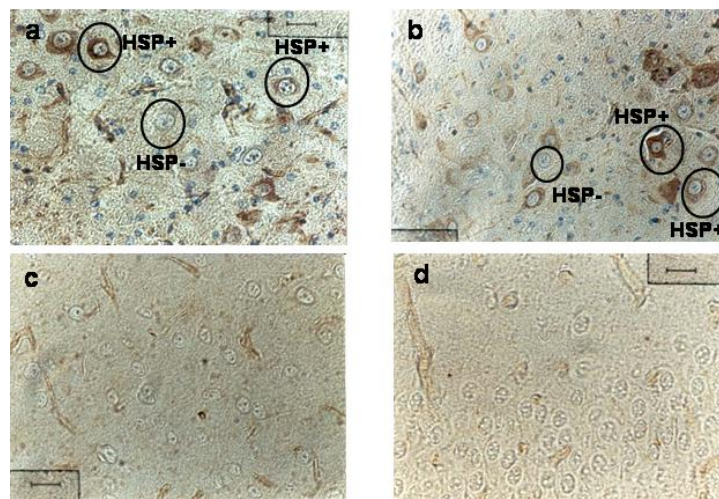

Figure 5- Photomicrographs of HSP- 27 immunostaining in brain stem (a, b) and striatum (c, d) of NGF treated (a, b) and untreated mice (c, d). Positive HSP immunoreactive (HSP- ir) neurons can be seen in brain stem but not striatum of treated and untreated mice. No differences in the intensity of HSP- ir can be seen after the NGF treatment.

Discussion

According to the literature, NGF is a neurotrophin able to mediate responses both in the immune and the nervous system (Bueker, 1948; Boyle *et al*., 1985; Aloe *et al*., 1997). Increased level of both NGF and cytokines in autoimmune diseases such as lupus erythmatosus and arthritis rheumatitis and in the acute phase of the multiple sclerosis and its animal model (Aloe *et al*., 1992; Bracci-Laudiero *et al*., 1992, 1993; Otten and Gadient, 1995), hints at a possible stimulatory effect of NGF on immune system. Overloading NGF in the central nervous system, we have tested if any immune response has been triggered by increased level of NGF. A triggered immune response would be recognized by an inflammation involving cell invasion and blood brain barrier leakage. Our histological examination of brain after the NGF infusion showed no signs of inflammatory responses. A second criteria for a triggered immune response would be to examine the levels of heat shock proteins which are known to act as autoantiogens (Aquino *et al*., 1997). This was also tested by our immunohistochemical studies which showed that following NGF treatment there is no changes in the level of HSP expression either in brain stem or other areas.

We therefore conclude that the ineffectiveness of the infused NGF on the immune system might be due to: Firstly that, NGF alone is unable to generate immune responses, requiring some additional factors to act synergistically; Secondly that, to stimulate immune responses different NGF regimen (time and dose) is required; Thirdly that, few single injections intracerebroventricularly is an inappropriate way of NGF delivery to the central nervous system. As cited in other published data (e.g. Farah, 2004), one appropriate and effective way of delivery is using osmotic pump which delivers miniature amounts of the drugs in each second. Unfortunately, due to inaccessibility and high prices of these pumps, we were unable to use them in these experiments. In future studies, we will try to find more appropriate ways of NGF infusion and application in the animal model of EAE which has successfully been generated in our lab currently.

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