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آموزش مهارت‌های کاربردی در تدوین و چاپ مقاله
Nerve Growth Factor Prevents Demyelination, Cell Death and Progression of the Disease in Experimental Allergic Encephalomyelitis

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ABSTRACT

Experimental allergic encephalomyelitis (EAE), a demyelinating disease induced in the animals parallels multiple sclerosis in human in several aspects, provides a useful model to investigate multiple sclerosis.

In this study, we have therefore used this model to study functions of nerve growth factor (NGF) in EAE. NGF with considerable effects on neuron survival, proliferation and differentiation of the nervous system, is also known to act on cells of the immune system. Simultaneous upregulation of proinflammatory cytokines and increased level of NGF points at possible effects of the nerve growth factor in autoimmune diseases.

To investigate roles of NGF in experimental allergic encephalomyelitis in vivo, we therefore decided to apply it intracerebroventricularly at a dose of 0.20 mg/mice prior to the induction of EAE.

Our clinical observations showed that in the EAE induced animals who received NGF, severity of the disease was reduced significantly compared to that in saline treated EAE mice. Also neuropathological investigation of spinal cords revealed that in contrast to saline treated EAE mice, no signs of cell death, infiltration and demyelination can be seen in NGF treated EAE mice, suggesting that NGF may have clinical implications in multiple sclerosis.

Key words: Experimental allergic encephalomyelitis; Myelin; Nerve growth factor; Spinal cord

INTRODUCTION

Multiple sclerosis is an inflammatory and demyelinating disease of the central nervous system. Experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis can be generated by immunizing different species of genetically susceptible animals using varieties of neuroantigens. Different types of neuroantigens such as myelin basic protein (MBP), proteolipoprotein (PLP) or myelin oligodendrocyte glycoprotein (MOG) are the most commonly used encephalitogenic peptides. Among these, MOG 35-55 is found to be a potent antigen which results in a progressive and severe type of the disease. The clinical course of the disease typically consists of an acute phase followed by a recovery phase and then a number of relapses and remissions. We hypothesized that immunization of C57/BL6 mice with a sequence
(35-55) of myelin oligodendrocyte glycoprotein would be the best choice.

Nerve growth factor (NGF) is a well characterized neurocytokine with potentials on survival, proliferation and differentiation of different types of neurons in peripheral and central nervous systems.\(^5\)\(^6\) NGF level increases in the cerebrospinal fluid (CSF) of MS patients during the acute phase of the disease and it decreases during the relapsing phase.\(^10\)\(^-\)\(^13\) NGF also has potentials to affect chemotaxis for polymorphonuclear cells of the immune system.\(^14\) NGF has been shown also to induce a decrease in IFN-\(\gamma\) which is involved in regulation of MHC molecules in neurons and glial cells of the brain and spinal cord in EAE rodents.\(^15\)\(^,\)\(^16\) These findings point to a dual interaction of NGF and the immune system which would be interesting to be investigated in multiple sclerosis. In the present study we sought to determine if intracerebroventricular application of NGF in EAE C57/BL6 mice has any impact on the disease.

**MATERIALS AND METHODS**

**Animals, Surgery and Treatments**

Female C57/BL6 mice (approximately 20 gr; 12 weeks old) were purchased from Pasteur Institute (Tehran, Iran), housed in groups of seven per Plexiglass cage (15×20×30 cm) and allowed free access to food and water.

To apply NGF intracerebroventricularly, mice were anaesthetized with an intraperitoneal injection of (100-150 \(\mu\)l) 5% chloral hydrate dissolved in 0.9% sterile saline. Mice were mounted in stereotaxic frame with lambda and bregma in the same horizontal plane. NGF dissolved in PBS was placed onto a gel foam called spongostan and then inserted into brain lateral ventricles with coordinates (+0.6 AP, -1.5 ML, +3.5 DV) taken from bregma according to the stereotaxic atlas of Franklin and Paxinos.\(^17\) Spongostan is a biodegradable matrix scaffold composed of gelatin and can be used in combination with delivery systems.

To induce EAE, mice were then immunized with 200 \(\mu\)g of MOG peptide (35-55) emulsified in complete Freund's adjuvant supplemented with 4mg/ml of killed mycobacterium tuberculosis. A volume of 0.1 ml of the mixture was injected at the base of the tail in each mouse. Pertussis toxin (500 ng/mouse) was injected immediately after and 48 hours later. Mice were grouped into three groups of seven including intact controls, saline treated EAE and NGF treated EAE. On day 10 post-immunization, mice were anaesthetized with chloral hydrate 5% and perfused transcardially with paraformaldehyde 4%.

**Assessment of the Disease**

EAE mice were weighed before and after saline or NGF treatment and assessed for clinical signs of disease on a daily basis. Clinical assessment was performed on the basis of the following scores: 0- normal, 1- limp tail or mild hind limb weakness, 2- limp tail and moderate hind limb weakness, 4- limp tail and severe hind limb weakness or moderate ataxia, 5- paraplegia with no more than moderate forelimb weakness, 6- limp tail and paraplegia with severe forelimb weakness or severe ataxia followed by death.\(^18\)

**Tissue Preparation**

Mice were perfused with paraformaldehyde 4% in 0.1 M phosphate buffer, pH 7.4, through the heart and postfixed in the same fixative for 24-48 hr. Using fine forceps, the L4–S1 segment of the spinal vertebrae was removed, and soaked in ethanol 70% until the day of embedding. For paraffin embedding, tissues were first dehydrated in 90%, 96%, 100% (2 changes) each for 1 hour and then cleared by incubations in ethanol/toluene (2:1) ethanol/toluene (1:1), toluene 100% (2 changes) of each for 30-45 minutes. Finally the embedding was started by toluene/paraffin (1:1) for half an hour and continued to four changes of paraffin baths each for 15 minutes. Blocks were allowed to be cooled and sectioned by using a rotary microtome at 7 \(\mu\)m. Sections were stained by Luxol fast blue (myelin stain) and Cresyl violet (nucleus stain) and mounted by depex.

**RESULTS**

**Clinical Signs**

As shown in figure 1, in contrast to saline treated, EAE mice in which clinical scores and therefore severity of the disease was progressive (from 1.1 at day 4 post immunization to 3.3 at day 9 post-immunization; \(p < 0.05\)), progressiveness of clinical scores was not statistically significant in NGF treated EAE mice (from 1.3 at day 4 post immunization to 2.7 at day 9 post-immunization; \(p > 0.1\)).
Neuropathology of the Spinal Cord

Light microscopy examination of Luxol fast blue stained spinal cords of untreated control, saline treated EAE and NGF treated EAE showed that there were neurons with highly dense chromatin reflecting pyknotic cells in the gray matter of saline treated EAE (Figure 2b) but not in NGF treated or untreated control spinal cord (Figure 2a, c). Also there were infiltrated cells in the meninges and around parenchymal venules and extensive vacuolization of myelin in white matter of the saline treated EAE spinal cord (Figure 2b) but not in NGF treated or untreated control spinal cord (Figure 2a, c).

Figure 1. A comparison between clinical scores in saline treated (n=7) and NGF treated EAE (n=7) mice from 4 to 9 days post immunization (DPI). While clinical scores and therefore severity of the disease were progressive in saline treated EAE (right panel; p<0.05), progressiveness of clinical scores were not statistically significant in NGF treated EAE mice (left panel; p>0.1).

Figure 2. Photomicrographs of cross sectioned spinal cords stained with luxol fast blue (a myelin stain) and cresyl violet (nucleus stain). (a) intact control, (b) saline treated EAE mice and c) EAE mice treated with NGF. Black arrows point at extensively stained pyknotic neurons (dead neurons) that are ubiquitously found in both dorsal and ventral horns of the spinal cord. Green arrows point to vacuolated myelin and red arrows point to the infiltrated cells in meninge of the spinal cord in untreated EAE animals (b). No pyknotic cells, vacuolated myelin or infiltrated cells is seen in NGF treated EAE spinal cord (c) or intact control (a). Also, note at the difference in stained myelin intensities in the intact control and NGF treated EAE spinal cords compared to that in saline treated EAE.
DISCUSSION

The present study demonstrates for the first time that in vivo application of NGF in EAE mice has significant inhibitory effects on neuron death, infiltration of immune cell and demyelination in the spinal cord which results in amelioration of clinical signs and progression of the disease. Amelioration of the clinical signs of EAE by NGF has also been shown by Villoslada and colleagues\(^{15}\) by using osmotic minipumps in marmosets.

Conversely, exacerbation of neurological signs has been occurred in NGF-immunized EAE rats.\(^{18,19}\) Increased cell death and axonal damage in a time-dependent manner following EAE induction has also been reported by Shields and colleagues\(^{20}\) which coincides with increased calpain expression and activity.\(^{21}\)

While our study provides the evidence for the first time that intracerebroventricular application of NGF blocks neuron death in spinal cord of in EAE mice, the results by Triaca and colleagues\(^{19}\) indicated that NGF also induced a regenerative response to neuronal damage occurring in the adult brain during EAE. They have shown that intracerebroventricular administration of NGF can act on growth and differentiation of brain precursor cells in the subventricular zone (SVZ) of brain in EAE rats.

Moreover, our results clearly showed that NGF infusion in cerebral ventricles of EAE mice abolished infiltration of immune cells in both white and gray matters of spinal cord. Effects of NGF on infiltration has also been shown in culture model of the endothelial blood-brain barrier (BBB), in which NGF directly interfered with blood-derived monocytes via its receptor, p75.\(^{22}\)

Moreover, Kramer and colleagues\(^{23}\) have shown that NGF gene transduction in nerve-specific autoimmune T lymphocytes has significant anti-inflammatory effect on infiltrating macrophages across blood brain barrier and can be used as vehicles to deliver therapeutically useful neurotrophic factors.

In the present study, we have also shown that NGF significantly abolished myelin vacuolization and destruction in EAE induced mice. In accordance with our in vivo results there is evidence, mostly in vitro, from other researchers which imply to the effect of NGF on myelination. Damarjian and colleagues\(^{24}\) have suggested that demyelination, axonal degeneration and dysregulated ion channel expression that contribute to the pathophysiology of EAE, are associated with the expression of p75 and trkA, NGF receptors, in the majority of Purkinje cells in cerebellum of EAE mice. Moreover, Du and colleagues\(^{25}\) have shown that NGF and other neurotrophins increase the in vitro expression of myelin basic protein, a myelin protein, in differentiating basal forebrain oligodendrocytes which express neurotrophin receptors, trk and p75. Also, Williams and colleagues\(^{26}\) have shown that myelin inhibitors not only inhibit axonal growth but also prevent neurotrophins from stimulating growth in cultured cerebellar neurons. Furthermore, Chan and colleagues\(^{27}\) have shown that NGF controls axonal receptivity to myelination by Schwann cells or oligodendrocytes in culture. Altogether these findings indicate that NGF may have therapeutic impacts on myelination in demyelinating diseases such as multiple sclerosis.

In conclusion, our study revealed that NGF infusion in vivo has anti-inflammatory effects and contributes to the recovery of EAE by preventing demyelination, cell death and immune cell infiltration.

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