Therapeutic effects of pegylated-interferon-α2a in a mouse model of multiple sclerosis

SANAZ AFRAEI1, REZA SEDAGHAT2, FARZANEH TOFIGHI ZAVAREH3, ZAHRA AGHAZADEH1, PARVIN EKHTIARI1, GHOLAMREZA AZIZI1,4, ABBAS MIRSHAFIEY1

1Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
2Department of Anatomy and Pathology, Faculty of Medicine, Shahed University, Tehran, Iran
3Non-Communicable Diseases Research Center, Alborz University of Medical Sciences, Karaj, Iran
4Research Centre for Immunodeficiencies, Paediatrics Centre of Excellence, Children’s Medical Centre, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Introduction: Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis (MS). EAE is mainly mediated by adaptive and innate immune responses that lead to an inflammatory demyelination and axonal damage. The aim of the present research was to examine the therapeutic efficacy of Peg interferon alpha 2a (Peg-IFNα-2a) as a serine protease inhibitor on EAE model.

Material and methods: EAE induction was performed in female C57BL/6 mice by myelin oligodendrocyte glycoprotein (35-55) (MOG35-55) in Complete Freund’s Adjuvant (CFA) emulsion, and Peg-IFNα-2a was used for the treatment of EAE. During the course of the study, clinical evaluation was assessed, and on day 21 post-immunisation blood samples were taken from the heart of mice for evaluation of IL-6, and enzymatic and non-enzymatic antioxidants. The mice were sacrificed and the brains and cerebellums were removed for histological analysis.

Results: Our findings indicated that Peg-IFNα-2a had beneficial effects on EAE by attenuation of the severity and a delay in the onset of disease. Histological analysis showed that treatment with Peg-IFNα-2a can reduce inflammation criteria. Moreover, in Peg-IFNα-2a-treated mice the serum level of IL-6 was significantly less than in controls, and total antioxidant capacity was significantly more than in the control animals.

Conclusions: These data indicate that Peg-IFNα-2a as an anti-serine protease with immunomodulatory properties may be useful for the treatment of MS.

Key words: experimental autoimmune encephalomyelitis, multiple sclerosis, antioxidant, Peg interferon alpha 2a.

Introduction

Multiple sclerosis (MS) is an autoimmune and inflammatory disease of the central nervous system (CNS), characterised by relapsing-remitting attacks and worsening neurologic function. MS manifests by demyelination and neurodegeneration among MS plaques that exist in the white matter [1]. Many clinical symptoms are known for MS, which include optic neuritis, diplopia, weakness, parasthesia or focal sensory loss, and ataxia [2]. MS might be transferred to an animal model defined as experimental autoimmune encephalomyelitis (EAE), through a mediation by autoantigen-specific T cells such as myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP). Researchers use EAE for pharmacological research and to evaluate the mechanisms that cause MS [3]. TCD4, TCD8, B cells, macrophages, and glial cells are involved in the pathogenesis of EAE [4]. Activation of these cells is responsible for increased production of pro-inflammatory cytokines and chemokines, which results in axonal damage and demyelination [4, 5]. They also produce ROS and RNS in CNS. The oxidative stress and inflammatory mediators play a key role in the pathogenesis of MS and EAE by damaging axons and oligodendrocytes [6].

Antioxidants reduce the expression of inflammation-associated molecules such as iNOS and nitrotyrosin, which is a marker of peroxynitrite reactivity in the CNS of EAE mice [6]. Previous studies showed that a lack of balance between antioxidant defence and ROS creates oxidative stress [7]. Antioxidant enzymes are among the most important defences against oxidative stress, which play a role in detoxification of ROS and removal of free radicals set
differently in MS. SOD is an antioxidant enzyme that act as a first line of defense against ROS, which that catalyses the dismutation of superoxide anion (O$_{2}^{-}$) to H$_{2}$O$_{2}$. This enzyme can be converted to H$_{2}$O and O$_{2}$ by catalase (CAT) and glutathione peroxidase (GPx) [8]. In other words, CAT and superoxide dismutase (SOD) act as synergistic enzymes. Mononuclear phagocytes are effector cells that cause demyelination in CNS by producing reactive oxygen species and damaging the blood brain barrier. SOD, CAT, Glutathione reductase (GR), and GPX enzymes protect CNS against ROS [9]. GR is another antioxidant enzyme that is vitally important to resistance against oxidative stress. GR catalyses the reduction of glutathione disulphide (GSSH) to the sulphydryl form of glutathione (GSH) [10-12], which is effective in preventing oxidative stress and can act as a scavenger for hydroxyl radicals, singlet oxygen, and various electrophiles. Activation of GR and SOD can be used as an indicator of oxidative stress [13]. IL-6 is one of the cytokines that mediates cellular responses during immune activation and inflammation, known as an important mediator of many inflammatory processes. It plays a major role in inflammatory reactions, neuroimmunology, and neuroinflammation [14]. Previous work shows that IL-6 is a regulator of Th17 differentiation in vitro, and anti-IL-6 can be applied for the treatment of EAE and CIA [15]. IL-6 and IL-17 are detected in chronic lesions of patients with MS [16]. Th17 cells have a direct role against self-antigens; therefore, they play a crucial role in the process of developing EAE and collagen-induced arthritis (CIA), as an experimental model of rheumatoid arthritis [17, 18].

In some experiments on humans, the reported levels of IL-6 have been increased in mononuclear cells in the blood and cerebrospinal fluid (CSF) [19-21] and in brain tissue of patients with MS using a double-label immunohistochemistry technique [22]. Furthermore, studies in both human MS patients [20] and in mouse models of MS (EAE) suggest that IL-6 levels may correlate with disease severity [23]. Other investigations showed that IL-6-deficient mice have been shown to be highly resistant to the induction of EAE [24].

In this research, our aim was to test the therapeutic efficacy of Peg interferon alpha 2a (Peg-IFN α-2a) in an experimental model of MS based on clinical assessment and histopathology, as well as evaluating the IL-6, total antioxidant capacity, enzymatic antioxidant parameters (including SOD), and glutathione reductase (GRx).

Material and methods

Animal selection and grouping

In this investigation, we used 16 female C57BL/6 mice, weighing 18-20 g, aged eight weeks, that were purchased from the Experimental Animal Centre of the Pasteur institute of Iran. Mice were housed according to institutional guidelines with access to food (pelleted diet) and water. Mice were randomly divided into three groups:

1 – normal group (healthy control, four mice), II – control group (six mice), and III – treatment (IFN) group (six mice). For adaptation, mice were kept in a temperature- and humidity-controlled environment in the animal house of Tehran University of Medical Sciences for two weeks. In this project, the same meal plan, including pelleted diet soy, peanuts, and water, was used. All procedures involving animals were performed according to the guidelines of Animal Ethics approved by Tehran University of Medical Science.

**Experimental autoimmune encephalomyelitis induction and treatment protocol**

All mice were weighed on the first day of the adaptation until the end of the experiment, and their weight was recorded. EAE induction was performed by Hook Kit (Hooke Laboratories, Inc., USA). Each kit contained two components: a vial of lyophilised pertussis toxin (PTX) and two pre-filled syringes consisting of MOG35-55 in an emulsion with Complete Freund’s Adjuvant (CFA). Hook Kite was used to induce EAE according to the guidelines; 0.1 ml MOG35-55 was injected subcutaneously into the upper back and then into the lower back to each mouse. After two hours, 0.1 ml PTX was injected intraperitoneally into each mouse. Moreover, after 24 hours, the second dose of PTX (0.1 ml/mouse) was injected intraperitoneally. A 180-microgram vial of Peg-IFN α-2a was purchased from F. Haffman-La Roche, Switzerland, and dissolved in 250 ml of saline solution. From the first day, 0.1 ml of Peg-IFN α-2a was injected subcutaneously to the IFN group. Total number injections per mouse was four and their interval was five days. The experiment was ended on day 21. Mice were checked daily for evaluating the effect of Peg interferon alpha 2a and the clinical score was assessed according to the following criteria: 0 – no clinical sign, 0.5 – paralysis of the tip of the tail, 1 – complete paralysis of the tail, 1.5 – complete paralysis of the tail and inhibition of hind legs, 2 – complete paralysis of the tail and numbness of the hind legs, hind legs coming together when lifting the mouse from the tip of the tail, 2.5 – complete paralysis of the tail, dragging the hind legs when moving, 3 – complete paralysis of the tail and hind legs and/or paralysis of tail and one leg and one paw, 3.5 – complete paralysis of the tail and hind legs and moving the mouse to the edge of the cage, 4 – complete paralysis of the tail and hind legs and partially paralysed paw, 4.5 – complete paralysis of the tail, hind legs, and paws, there is no movement in the mouse, and 5 – killing the mouse is proposed at this stage.

**Evaluation of histopathology and in vitro determinants**

On the 21st day after induction, all mice in normal, control, and IFN groups were first anaesthetised by chloroform and a blood sample was taken from the right ventricle of the heart immediately by splitting the chest. In order to obtain
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serum with high quality, blood samples were centrifuged at room temperature and sera were separated from blood. All sera were stored at −20°C until the time of TAC, IL-6, SOD, and GRx assay. For fixation and removal of blood cells of the brain and spinal cord, perfusion of the heart was administered. Brain and cerebellum were separated and fixed in 10% formalin. Cross sections (5 μm) of brain and cerebellum were prepared and embedded in paraffin, and stained with haematoxylin-eosin (H&E) to evaluate the inflammatory criteria and leukocyte infiltration intensity. Also sectioning (8 μm thick) for Luxol fast blue (LFB) was done to detect demyelination. Finally, stained slides were evaluated by an expert pathologist blind to the study.

Quantification of super oxide dismutase activity

A ZellBio GmbH SOD kit (Ulm, Deutschland) was used to assay the SOD in sera of mice in all groups. It can be used for SOD activity determination in the range of (5-100 U/ml with 1 U/ml sensitivity). In this assay, the SOD activity unit was considered as the amount of sample that will catalyse decomposition of 1 μmol of O2− to H2O2 and O2 in one minute. The final activity of SOD was determined calorimetrically at 420 nm.

Glutathione reductase assessment

A zellbio GmbH (Ulm, Deutschland) kit to assay GR was used. Biocore GR assay kit can be used for activity determination in the range of 10-15 U/l with 1U/l sensitivity. The GR activity was determined photometrically at 340 nm.

Total antioxidant capacity

Measurement of total antioxidant capacity in serum with colorimetric method by radical cation of 2,29-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was introduced by Miller and Rice-Evans. The experiment is based on eliminate or revive of cation ABTS + (with a maximum light absorption at wavelengths of 660, 734, and 820 nm) by antioxidant compounds in serum sample. With reviving radical ABTS, green-blue solution turns into achromatic solution. The decrease in optical density measured by a spectrophotometer and expressed as radical inhibition percentage. Instability of ABTS is a weakness of this method, which is improved by production of stable ABTS. To produce ABTS cation (2,2 azino-bis(3-Ethyl-Benzolin-6-Sulfonicacid)) it is combined with potassium persulphate that is stable for at least two days. In this investigation, Bovine Serum Albumin (BSA) was manipulated instead of Trolox. For converting the inhibition percentage to gr/dl, standard BSA curve was used.

Quantification of interleukin-6

We used ELISA assay to test the level of IL-6 in the serum of mice in control, normal, and treatment groups by a sandwich Biolegend LEGEND MAX Mouse IL-6 ELISA Kit (Biolegend, Inc., San Diego). The kit consists of a 96-well strip plate that is pre-coated with a capture antibody that was used to assay the level of pro-inflammatory IL-6. This assay was performed according to the manufacturer’s instructions. Absorbance was read at 450 nm in a 96-microplate ELISA reader.

Statistical analysis

Data were expressed as mean ± SD, except for histological scores, which were calculated as mean ± SEM. Statistical analysis was performed with the Mann-Whitney U-test for nonparametric data and Student’s t-test for parametric data. A p value < 0.05 was considered statistically significant.

Results

Clinical findings

In this experiment, EAE was induced in C57BL/6 mice by immunising them with a ready-to-use Hooke kit. Mice were dosed subcutaneously with Peg-IFN-α-2a in the IFN group. The mice in this group showed significant reductions in the clinical course of EAE compared to the control group (Fig. 1). *p < 0.05. Also, EAE onset was delayed in the IFN group (11.50 ±1.22), compared to the control mice (10.5 ±0.54), *p < 0.01 (Fig. 2). These effects indicate that Peg-IFN-α-2a can inhibit the progression of EAE. There were no manifest toxicities in any mice receiving Peg-IFN-α-2a.

Histological findings

The aim of this research was to discover the correlation between the clinical symptoms of EAE with histopathology of CNS in control and Peg-IFN-α-2a-treated mice. Histological analysis was performed by LFB and H&E staining on brain and cerebellum in EAE mice receiving subcutaneously Peg-IFN-α-2a or vehicle. An expert pathologist scored all sections by light microscopy. Representative images of LFB- and H&E-stained tissue sections from all groups illustrated that demyelination and inflammation determinants in EAE mice treated by Peg-IFN-α-2a were significantly lower than control group (Fig. 3). The results in Table 1 and 2 show that the clinical intensity of EAE in the control and IFN group is correlated with intensity of inflammation observed in histopathology of CNS.

Super oxide dismutase activity

As shown in Figure 4, SOD activity was increased in the treatment (29.37 ±16.33) group, compared to the control mice (20.51 ±4.77), (p = 0.351). In the normal group, SOD activity was (20.97 ±1.99). There was no significant difference between the normal and control groups. Peg-IFN-α-2a therapy showed a non-significant increase
in SOD activity in serum, which was in agreement with the clinical findings.

Glutathione reductase

As shown in Figure 5, GR activity was increased in the IFN group (44.20 ± 30.81) compared to the control mice (20.18 ± 3.36) (p = 0.099). In the normal group, GR activity was significantly higher than that of the control group (p < 0.001). Peg interferon 2a therapy showed an increase in GR activity in serum, which was in agreement with the clinical findings.

Total antioxidant capacity evaluation

TAC evaluation is based on ABTS radical cation (2,2-azino-bi(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging on serum samples of mice. As shown in Figure 6, treatment with Peg interferon-2a significantly increased TAC (2.25 ± 0.49) compared to the control group (1.68 ± 0.22) (p = 0.041).

Interleukine-6 evaluation

The effect of Peg-IFN α-2a on IL-6 cytokine concentrations of mice sera was evaluated. The analysis was performed using an ELISA kit. As shown in Figure 7, treatment by Peg-IFN α-2a reduced IL-6 production in the treatment group (90.94 ± 15.60) compared to the control group (138.65 ± 37.35) (p = 0.046).

Discussion

EAE is an animal model of MS, which causes brain inflammation and demyelination mediated by immune system response to brain antigens [25]. Th1 and Th17 and their proinflammatory cytokines including TNF-α, IFN-γ, and IL-17 along with myelin-specific CD8+ T cells and infiltrated macrophage within the CNS are assumed to be important mediators for disease induction [26]. Immunomodulatory agents are reasonably effective in the treatment of MS and EAE, and cause a delay in the progression of disabling the patient [27-31].

Peg-IFN α-2a was developed in an attempt to improve the pharmacological profile of IFN α. Covalent attachment of a branched 40-kd polyethylene glycol moiety to IFNa-2a results in more sustained absorption (time to peak
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Fig. 3. Representative light microscopic view of histopathological slides of central nervous system in different groups. A) H&E staining of brain sections showed that Peg-IFN α-2a therapy could suppress the progression of inflammation significantly by restricting leukocyte infiltration. B) LFB staining showed that Peg-IFN α-2a could decrease the extent of demyelination. N – normal, C – control, I – Peg-IFN α-2a treated

Table 1. Comparison of inflammation items in histopathology examination in the brain of experimental autoimmune encephalomyelitis mice

| Inflammation criteria                        | Control (Mean ±SD) | IFN (Mean ±SD) | p-value* |
|---------------------------------------------|--------------------|----------------|---------|
| Demyelination                               | 3.85 ±0.74         | 1.50 ±1.41     | 0.012   |
| Degeneration                                | 3.70 ±0.57         | 1.45 ±1.33     | 0.012   |
| Infiltration of inflammatory cells in leptomeninges | 3.50 ±0.64         | 1.30 ±1.30     | 0.021   |
| Meningeal vessels hyperaemia                | 3.50 ±0.61         | 1.80 ±1.68     | 0.055   |
| Leukocyte margination                        | 2.65 ±0.60         | 1.30 ±1.30     | 0.012   |
| Perivascular cuffing                        | 3.65 ±0.85         | 1.30 ±1.30     | 0.015   |
| Perivascular oedema                         | 3.20 ±0.57         | 1.10 ±1.08     | 0.011   |
| Hypercellularity                             | 4.11 ±0.70         | 1.70 ±1.56     | 0.008   |
| Laminar necrosis                             | 3.90 ±0.65         | 1.60 ±1.55     | 0.017   |
| Endothelial cell hypertrophy                 | 3.60 ±0.82         | 1.20 ±1.09     | 0.008   |
| Spongiosis                                   | 3.05 ±0.67         | 1.25 ±1.09     | 0.15    |

Source: histopathology examination.

0 = no symptoms, 1 = mild, 2 = moderate, 3 = moderately severe, 4 = severe, 5 = very severe. Demyelination: loss of myelin from the nerve sheaths, leptomeninges: the two innermost layers of tissue (the arachnoid mater and pia mater) that cover the brain and spinal cord, hyperaemia: too much blood that accumulates in micro circulation as meningeal vessels, leukocyte margination: accumulation and adhesion of leukocytes to the endothelial cells of blood vessel walls at the site of injury in the early stages of inflammation, perivascular cuffing: the accumulation of leukocytes around the vessel, laminar necrosis: necrosis of the selected neuron in particular layer of cerebral cortex, spongiosis: vacuolation of cerebral grey and white matter, hypercellularity: increased number of glial and/or inflammatory cells in a tissue.

*All differences were statistically significant (*p < 0.05) except for meningeal vessels hyperaemia.
plasma concentration increased), reduced clearance, and a smaller volume of distribution. PEG-IFN is an immuno-modulatory agent that can induce intracellular antiviral activity and inhibit the proliferation process [32, 33]. The combination therapy of (PEG-IFN) and ribavirin for HCV infection had many side effects [34]. Monotherapy with Peg-IFN \(\alpha\)-2a may be used for patients with a contraindication to ribavirin. It was reported the protease inhibitors such as boceprevir, with antiviral activity, can be used in combination with Peg-IFN \(\alpha\)-2 and ribavirin for greater efficacy [35]. Cytokines, chemokines, and proteases are the effector agents promoting demyelination and axon injury in multiple sclerosis. To date, proteases are the attractive targets for development of new drugs for treatment of a variety of autoimmune diseases such as MS [36]. These results allow us to predict that Peg-IFN \(\alpha\)-2a can suppress the onset of symptoms and severity of EAE in C57BL/6 mice. In this investigation we evaluate the efficacy of Peg-

**Table 2.** Comparison of inflammation items in histopathology examination in the cerebellum of experimental autoimmune encephalomyelitis mice

| Inflammation criteria                          | Control (Mean ±SD) | IFN (Mean ±SD) | p-value* |
|-----------------------------------------------|--------------------|----------------|----------|
| Demyelination                                 | 3.75 ±0.66         | 1.70 ±1.6      | 0.025    |
| Degeneration                                  | 3.80 ±0.45         | 1.50 ±1.36     | 0.007    |
| Infiltration of inflammatory cells in leptomeninges | 3.50 ±0.79      | 1.10 ±1.14     | 0.012    |
| Meningeal vessels hyperaemia                  | 3.80 ±0.76         | 1.55 ±1.41     | 0.008    |
| Leukocyte margination                         | 3.90 ±0.89         | 1.50 ±1.41     | 0.015    |
| Perivascular cuffing                          | 3.80 ±0.76         | 1.20 ±1.30     | 0.011    |
| Perivascular oedema                           | 3.15 ±0.86         | 1.20 ±1.15     | 0.02     |
| Hypercellularity                              | 4.00 ±0.79         | 1.90 ±1.74     | 0.033    |
| Endothelial cell hypertrophy                  | 3.25 ±0.50         | 1.20 ±1.15     | 0.009    |
| Spongiosis                                    | 3.30 ±0.86         | 1.25 ±1.20     | 0.026    |

*All differences were statistically significant (*p < 0.05)

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**Fig. 4.** The effect of Peg-IFN \(\alpha\)-2a on serum SOD activity. It was observed that treatment with Peg interferon-2a increased SOD activity compared to control group, but this difference was not statistically significantly \((p = 0.351)\).

**Fig. 5.** The effect of Peg-IFN \(\alpha\)-2a on serum GR activity. It was observed that treatment with Peg interferon-2a increased GR activity compared to the control group but was not statistically significant \((p = 0.099)\). However, the difference between normal group and control group was statistically significantly \((p < 0.001)\).
IFN α-2a in EAE. It has been found that Peg-IFN α-2a, which is an antiviral, anti-proliferative, and immunomodulatory agent, can suppress the onset of symptoms and severity of EAE in C57BL/6 mice. It has been observed that using intraperitoneal application of Peg interferon α-2a in five-day intervals can significantly reduce the severity of inflammation determinants, such as demyelination, infiltration of inflammatory cells, neuronal degeneration, perivascular cuffing in the brain, and cerebellum of EAE mice treated with Peg-IFN α-2a, compared to the vehicle mice. Furthermore, studies in human MS patients [20] and in EAE [37] suggest that IL-6 levels might be related to the disease severity. We found that the treatment with Peg-IFN α-2a can reduce the level of IL-6 in EAE mice. This result is also consistent with our clinical and histopathological findings.

Several studies have demonstrated a significant increase in lipid peroxidation products in the brain, plasma, and cerebrospinal fluid in MS patients [38, 39]. Oxidative stress characterised by excessive production of ROS, and reduction of antioxidant defence mechanisms, are known to be implicated in the pathogenesis MS [28, 29, 40, 41]. The impairment of antioxidant systems or an increase in the production of ROS could contribute to lipoprotein peroxidation in MS. Lipoprotein lipid peroxidation products are neurotoxic and have proinflammatory properties, which can be involved in demyelination and axonal injury MS [39]. SOD and GR are the enzymes of antioxidant defence systems that are necessary for resistance against oxidative stress. In this investigation, SOD and GR enzymes were increased in treated mice by Peg-IFN α-2a because ROS may be removed by antioxidant defence such as SOD and GR [42]. In this study there were no differences between SOD activity in the normal and control groups. Rheumatoid arthritis (RA), as an autoimmune disease similar to multiple sclerosis, is associated with [43, 44] ROS as mediators of tissue damage in patients with RA and MS [45]. Antioxidant systems are destroyed by free radicals from oxygen metabolism [46]. Many researchers have shown that antioxidant enzymatic systems and/or non-enzymatic systems in RA are impaired [47]. Oxidative stress is the outcome of high levels and/or inadequate removal of ROS [46]. GR converts oxidised glutathione to reduced glutathione. These enzymatic and non-enzymatic antioxidants also play an essential role in inhibiting inflammation [48]. Other studies showed that GR is decreased in autoimmunity disease such as rheumatoid arthritis [19, 49]. Also, TAC is increased in mice that have been treated with Peg-IFN α-2a. Other experiments showed that treatment with pegylated interferon alpha increases TAC [50]. IL-6, as a pro inflammatory cytokine that is an important mediator of immune responses, inflammation, induction of the acute phase response, and differentiation of lymphocytes and monocytes, might enhance the pathogenesis of MS [51, 52]. Several studies have reported an increased level of IL-6 in mononuclear cells in the blood and cerebrospinal fluid (CSF) [19, 21], and in brain tissue of patients with MS [22]. Our result showed that pegylated interferon alpha can reduce IL-6 production in EAE mice. Finally, these data indicate that Peg-IFN α-2a therapy can attenuate the disease progression in experimental model of MS, and may be a useful approach for treatment of MS through inhibiting the production of a wide range of serine protease by glial cells.

The authors declare no conflict of interests.
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