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Interferon-beta-1beta protects against multiple sclerosis-induced endothelial cells apoptosis

Shaghayegh Haghjooy Javanmard [1], Mohammad Saadatnia M[2,3], Vida Homayouni V[1], Mahin Nikoogoftar M[4], Amir H. Maghzi[3,5], Etemadifar M[3], Chaitanya VG[6], Jeanie C. McGee[7], Alireza Minagar[7], J. Steven Alexander[6]

[1]Applied Physiology Research Center, Department of Physiology, Isfahan University of Medical Sciences, Hezar Jerib Avenue, Isfahan, Iran, [2]Isfahan Medical Education Research Center, Isfahan University of Medical Sciences, Isfahan, Iran, [3]Isfahan Neurosciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran, [4]Department of Hematology, School of Medicine, Tarbiat Modarres University, Tehran, Iran, [5]Neuroimmunology unit, Centre for Neuroscience & Trauma, Blizard Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, London, UK Departments of Molecular and Cellular Physiology[6] and Neurology[7], Shreveport, USA

TABLE OF CONTENTS

1. ABSTRACT

Disruption of the blood-brain-barrier (BBB) due to endothelial cell (EC) injury is an essential step in formation of multiple sclerosis (MS) lesions. We investigated the role of endothelial cell (EC) apoptosis in the pathophysiology of MS, studying the therapeutic effect of IFN-beta-1b against MS sera-induced endothelial apoptosis. Human umbilical vein endothelial cells were treated with sera from patients with active MS (in relapse), MS in remission, or sera from healthy volunteers (each n = 5). Apoptosis was assessed by annexin V-propidium iodide staining. Effects of IFN-beta-1b on EC apoptosis were tested at increasing doses (10, 100, and1000 U/ml). Nitrite (NO_2^-) levels were determined in culture supernatants. EC apoptosis was increased by sera from exacerbating MS patients, but not remission, compared to healthy individuals ($p<0.001$). Effects were blocked by IFN-beta-1b at 10U/ml ($p<0.05$), but not higher doses, and was associated with increased NO/NO_2- production ($p<0.05$). EC apoptosis leading to disruption of the BBB may play a role in MS etiology and represents a novel therapeutic mechanism of action for IFN-beta-1b in MS therapy.

2. INTRODUCTION

Multiple sclerosis (MS) is a relapsing demyelinating and neurodegenerative disease of human central nervous system (CNS) (1), which mainly affects young adults. Currently, the molecular basis of MS relapses and remissions are only partially understood and it is believed that the pathogenesis of MS involves both immune system and endothelial cells' responses, most of which remain to be elucidated (2, 3). While the initial event(s) which precipitates the pathophysiology of MS remains elusive, it has been hypothesized that interactions between environmental and genetic factors play essential roles as triggers in development of MS (1). There are several proposed models for MS pathophysiology, one of which is now known as the "vascular scheme" (3). This theory proposes that disruption of the blood brain barrier (BBB) caused by cerebral endothelial cell (CEC) dysfunction is a critical early event which allows for transendothelial migration of activated CNS-autoreactive immune cells into the CNS environment and subsequent formation of perivenular demyelinating MS lesions (2,3).

The integrity of the BBB is essential for normal CNS homeostasis and functioning. CEC injury or dysfunction has been associated with many acute and chronic neuroinflammatory diseases. *In vitro* studies have demonstrated that many stimuli can induce apoptosis (programmed cell death) in endothelial cells, suggesting that CEC apoptosis is an important mechanism underlying CNS vascular injury, which leads to diminished barrier, immune cell penetration of the CNS, inflammation, and coagulation (2,3). In the present study, we examined the role of endothelial cell apoptosis in the pathophysiology of MS. Although endothelial cell apoptosis can be observed in many inflammatory and immune-mediated disorders, this is the first study which provides direct experimental evidence linking endothelial cell apoptosis as a basis of MS pathogenesis and response to IFN-beta therapy.

3. METHODS

3.1. Patients and control subjects

The study was approved by the Ethics Committee of Isfahan University of Medical Sciences and study subjects provided signed consents. Ten patients with MS were recruited from Isfahan MS society (the details of this database have been previously published) (3). Newly diagnosed (<3months) treatment-naïve patients with relapsing-remitting MS (according to McDonald's criteria) were enrolled. Patients were divided into two groups: (i) patients with clinical relapse and at least one gadolinium-enhancing lesion on brain MRI and (ii) patients in remission defined as being in clinical remission with no contrast-enhancing lesions on their brain MRI. Five healthy age and sex-matched volunteers with no history of any diseases or neurological symptoms served as the control group. Venous blood specimen was drawn into 10 ml serum tubes without additives. After centrifugation, serum aliquots were frozen at -80°C until analysis.

3.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) (National Cell Bank of Iran affiliated with the Pasteur Institute, Tehran, Iran) were cultured in endothelial basal medium (EBM) supplemented with endothelial cell growth factor, gentamicin, amphotericin B, and 10% fetal calf serum (FCS) and expanded until the 3rd passage (P3) before experiments were performed.

For assessment of apoptosis, 10^5 cells were plated in 35-mm culture dishes. 24 hours before flow cytometry studies, the complete culture medium containing fetal calf serum was removed and cells were washed 1X with endothelial basal medium (minus FCS and other supplements) for 2 minutes. Culture medium containing 10% human control or MS serum +/- specified concentrations of IFN-beta 1b (Betaseron, Berlex) was then added for 24 hours. After 24 hours of incubation cells these cells were analyzed by flow cytometry as described below.

We studied the following sera added to HUVEC culture medium: (1) sera from healthy individuals (2) sera from MS patients (in relapse); (3) sera from MS patients (in clinical remission); (4) sera from MS patients (in relapse) + IFN-beta-1b (at 10 U/ml, 100 U/ml or 1000 U/ml).

3.3. Apoptosis analysis

For each stained cytometry sample, a total number of 10^5 cells were washed with ice-cold PBS once and were stained with annexin –PI as follows: cells (10^5/ml) were incubated with 1 µl annexin V-fluorescein isothiocyanate and 0.5 µl propidium iodide (PI, 10 mg/ml) in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$). Subsequently the cells were analyzed by fluorescence-activated cell sorting (FACScan, Becton–Dickinson). Apoptotic cells were designated as annexin-V^+/PI^- cells. Data were analyzed by Cell Quest software. Apoptosis quantification by Annexin V/PI assays is illustrated in Figure 1**.**

3.4. Nitric oxide (NO) metabolite (Nitrite, NO_2^-) measurement

Nitrite (NO_2-), an important NO metabolite in culture supernatants was determined using the Griess reaction (Parameter TM, total NO Assay kit, R&D Systems, USA) according to manufacturer's instructions. Briefly, in this assay, nitrite is detected colorimetrically as an Azo dye product of the Griess Reaction. The Griess Reaction is based on the two-step diazotization reaction in which acidified NO_2- produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophore azo-derivative, which absorbs light at 560 nm. Values were calculated using a standard curve produced with sodium nitrite.

3.5. Statistical analysis

Data were tested for normal distribution by the Kolmogorov-Smirnov test. Significance of variations between groups was evaluated using Kruskal Wallis test and by Mann Whitney test.

4. RESULTS

Five MS patients during relapse (mean age=33.2 ± 2.3, female to male (F:M) =3:2), five patients in relapse (mean age=32.1 ± 3.5, F:M =3:2), and five healthy control subjects (F:M=3:2) participated in this study. Treatment of HUVECs with the sera of untreated MS patients in clinical relapse for 24 hours resulted in significantly greater apoptosis than either sera of the patients in clinical remission or healthy controls ($p<0.001$, Figure 2). There were no significant differences in the levels of apoptosis in HUVECs between patients with MS in clinical remission compared with healthy volunteers ($p>0.05$).

We tested the effects of IFN-beta-1b on endothelial cell apoptosis in three doses (10 U/ml, 100 U/ml or 1000 U/ml). The addition of IFN-beta-1b suppressed the induction of apoptosis by the serum of patients in relapse only when used in doses of 10 U/ml, but not with higher doses (Figure. 3).

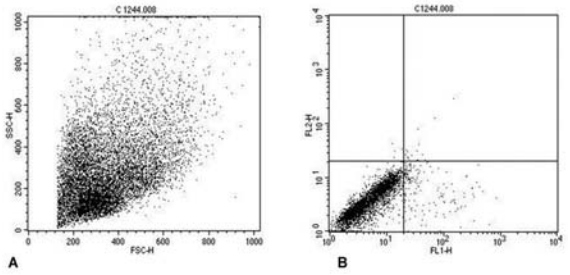

Figure 1. Quantitation of Apoptosis by Annexin V/PI assays. A: FSC versus SSC displays; B: Annexin V-FITC versus PI displays.

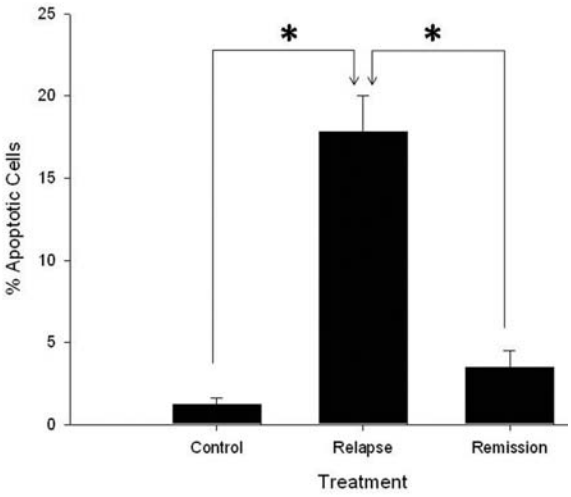

Figure 2. Induction of apoptosis in HUVECs by the serum of patients with MS (both un-treated in relapse or with MS in clinical remission) vs. healthy volunteers (control group). Apoptosis rates of HUVECs were measured after exposure to media containing 10% serum of patients with MS or control group for 24 h. *p < 0.001. Data are presented as the mean value ± SD

The measurement of nitrite concentration showed significantly greater levels of dissolved NO_2-/NO_3 metabolite in the culture media of HUVECs treated by 10 U/ml of IFN-beta-1b ($p<0.05$), while there were no significant differences between the two other doses and patients sera alone (Figure 4).

5. DISCUSSION

In the present study, we found that the level of apoptosis of human endothelial cells was significantly increased following incubation of these cells with sera from MS patients in exacerbation, (but not in sera from patients in MS remission) compared to healthy individuals. Most importantly, these effects are observed in the absence of immune cells, suggesting that stable circulating factors released during MS can affect the cerebral vasculature independently of immune cell mediated effects. These pro-apoptotic effects of sera from MS patients in exacerbation were significantly reduced by co-incubation with IFN-beta-1b at 10 U/ml, but not at higher doses. This protective effect of IFN-beta-1b was correlated with increased nitric oxide production at low IFN-beta-1b levels.

5.1. Endothelial dysfunction in MS

Disruption of the BBB permitting transendothelial penetration of activated leukocytes into the CNS is among the earliest event seen in the brain in MS, which results from inflammatory interactions between activated immunocompetent cells with CECs, their associated astrocytes, neurons, and oligodendrocytes (4, 5). Although the mechanisms responsible for the breakdown of the BBB in MS remain only partially understood, we previously reported on the direct effects of cytokines/chemokines on endothelial junctional dysregulation as well as indirect cytokine/chemokine-dependent leukocyte mediated injury via MMPs (6, 7). Within the CNS, antigen-presenting cells and Th1-type lymphocytes release large amounts of proinflammatory cytokines, particularly IFN-gamma, TNF-alpha, and IL-1-beta into the circulation, which act either alone or cooperatively to disturb the BBB by several mechanisms, including disorganization of BBB junctions, increasing leukocyte endothelial adhesion and migration, enhanced expression of class II MHC and shedding of endothelial 'microparticles' (EMP) during induction of endothelial cell apoptosis (4). Indirect cytokine/chemokine-dependent leukocyte mediated injury in MS patients has been demonstrated previously (5-10). However, few if any studies directly link effects of such cytokines/chemokines on endothelial stress and survival in MS patients. In this study we demonstrated increased apoptosis of HUVECs following incubation of these cells with sera from MS patients, demonstrating that factors present in MS sera are pro-apoptotic for endothelial cells. Most importantly, because this study was conducted in the absence of leukocytes, factors present in MS sera can promote endothelial injury independent of the action of leukocytes. Therefore, although leukocyte-dependent tissue injury is a well described phenomenon, pro-apoptotic serum factors may exacerbate or mediate endothelial cell apoptosis in active MS. Our data show that apoptosis with sera of MS patients in remission phase and relapse phase is 3 and 14 fold greater respectively than in the control group. This means that the rate of endothelial cell apoptosis in the 'relapse' phase of MS is still ~4 times greater than in the remission phase. More than 30 potential inducers of endothelial cell apoptosis have been identified, including neurohormones, cytokines, and newly discovered genes and 2nd messengers, therefore identifying endothelial active pro-apoptotic mediators in MS may provide important new targets in MS (10-13).

5.2. IFN-beta and apoptosis

IFN-beta remains the 'first line' treatment for MS patients, although its exact mechanism(s) of action remain unknown. Several mechanisms for IFN-beta protection have been proposed, including a classical anti-inflammatory mechanism, i.e. IFN-beta may shift polarization of the immune system from a pro-inflammatory (Th_1) to an anti-inflammatory state characterized by proliferation of type 2 (Th_2) T helper cells.

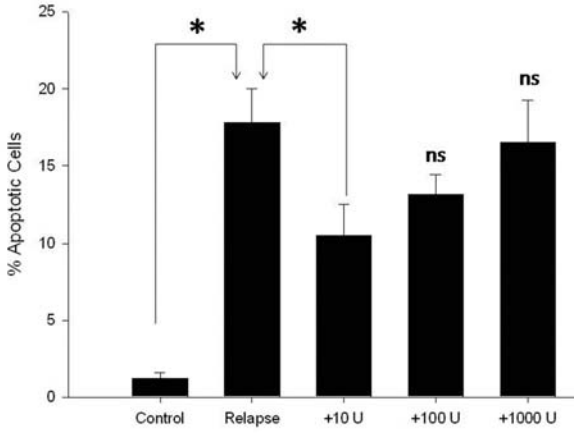

Figure 3. Suppression of MS serum-induced apoptosis by IFN-beta(10 U/ml, 100 U/ml, 1000 U/ml).Effect of IFN-betatherapy on proapoptotic activity of patient serum (n=5 per group). Data represent apoptosis rates in HUVEC incubated with patient serum for 24 hours +/- specific concentrations of IFN-beta quantified by flow cytometry. Dose-dependent inhibition of apoptosis by IFN-beta was only significant at 10 U/ml. *p< 0.05 vs. patient.

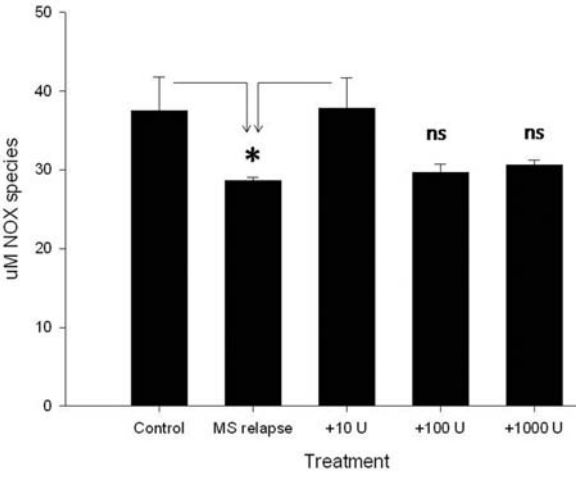

Figure 4. Effect of IFN-beta apoptosis in HUVECs. HUVECs were treated with sera from MS patients (untreated in relapse) +/- IFN-beta (10 U/ml, 100 U/ml, 1000 U/ml) and normal healthy controls, the concentration of nitrite in the culture medium was determined after 24 h. *p< 0.05 vs. patient.

Secondly, IFN-beta downregulates MHC II leading to decreased Th_1 activation with decreased IFN-gamma release from activated T cells (13- 15). IFN-beta also increases T cell apoptosis through inhibition of FLIP, an anti-apoptotic protein and inhibitor of the expansion of T cell clones. IFN-beta exposure of activated T cells in early MS may promote production of the suppressive Th_2 cytokine IL-10 which limits Th_1 responses (16-18). Other molecular mechanisms may also be involved in these IFN-beta responses. In patients with MS, IFN-beta therapy changes gene expression in several cell types via changes in transcription factors and mRNA activation. Although many intracellular molecular signaling pathways may participate in IFN-beta action, elucidation of these signaling mechanisms may be an important means of linking MS etiology and therapy (19-21). Lastly, IFN-beta may enhance the integrity of the BBB. IFN-beta can reduce BBB permeability to immune cells and proteins via (1) inhibition of MMP production by T-cells, preventing disintegration of the inter-endothelial junctions and the subendothelial matrix to restrict passage of active T cells into the inflamed CNS (21- 23), (2) releasing soluble vascular cell adhesion molecule (VCAM-1) that binds to activated T cells as a 'decoy' also preventing them from penetrating the CNS, and (3) lastly by exerting a direct stabilizing effect on endothelial cells *in vitro* to block their release of microparticles into the CNS (22-27).

In the current study we also provide evidence supporting direct stabilizing effects of IFN-beta-1b on the endothelium, which shows that besides the other properties ascribed to IFN-beta, it may preserve the integrity of BBB by reducing endothelial apoptosis. Previous *in vivo* studies demonstrated some anti-apoptotic properties for IFN-beta (13-15). IFN-beta can promote the elimination of auto-reactive B and T cell through the reduction of apoptotic proteins. However, there are few *in vitro* data for anti-apoptotic effects of IFN-beta. This study for the first time showed that IFN-beta-1b *in vitro* directly attenuates endothelial apoptosis.

5.3. Dose-dependent effects of IFN-beta-1b

In our study the effects of IFN-beta-1b in decreasing apoptosis were found to be dose-dependent and limiting. Previous *in vivo* studies (27-29) also showed dose-dependent effects of IFN-beta-1b. We found that IFN-beta-1b used a concentration of 10 U/ml decreased apoptosis more effectively than higher doses (100 U/ml and 1000 U/ml). Persistent, high levels of cytokine receptor occupancy can lead to 'tachyphylaxis', a state characterized by reduced biological sensitivity to agonists reflecting decreased receptor signaling through several possible mechanisms, including increased receptor turnover, decreased synthesis of receptor components, receptor proteolysis and desensitization to downstream signaling (30-33). Additionally, the receptor subunit composition of interferon receptors may also be influenced by receptor binding, particularly that of IFNAR-2c (the full-length trans-membrane IFNAR-2) and formation of soluble IFNRA-2a, a soluble decoy isoform (33, 34). Using decreased MxA expression as a marker of IFN-beta-1b action, clinical reports of tachyphylaxis in IFN-beta therapy have been described (35, 36). Therefore, low levels of IFN-beta-1b may elicit effective signaling, while persistent, high levels of IFN-beta-1b might diminish receptor expression and sensitivity to IFN-beta-1b.

5.4. Correlation of nitric oxide production with reduction in apoptosis

We have observed that reduced apoptosis was positively correlated with increased abundance of NO metabolites. It has been demonstrated that NO can inhibit apoptosis in a number of cell types including endothelial

cells (37, 38). NO can inhibit apoptosis through lowering mitochondrial respiration, cGMP/PKG mediated inhibition of permeability pore transition and by increasing mitochondrial membrane potential (38, 39). Since mitochondria contain pro-apoptotic proteins such as Apoptosis Inducing Factor (AIF), Smac/DIABLO, and cytochrome C, which are released through these mitochondrial pores, and lead to the formation of the apoptosome complex and the activation of the caspase cascade, NO may suppress apoptosis through one or more of these mechanisms (39, 40) Currently, stabilization of mitochondrial respiration seems to be an important potential mechanism underlying IFN-beta protection.

In conclusion, our results suggest a novel mechanism of therapeutic action for IFN-beta-1b, through its stabilization of the BBB by reducing endothelial apoptosis. Further studies are required to investigate the mechanisms underlying MS sera induced apoptosis to identify the apoptotic mediators present in the sera of MS patients and to elucidate the basis of IFN-Beta-1b mediated protection against.

6. ACKNOWLEDGEMENTS

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Send correspondence to: J. Steven Alexander, Department of Physiology, Alireza Minagar, Department of Neurology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130, Tel:318-675-4151, 318-675-4679, Fax: 318-675-4156, 318-675-6382, E-mail: jalexa@lsuhsc.edu

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