Minocycline up-regulates the expression of brain-derived neurotrophic factor and nerve growth factor in experimental autoimmune encephalomyelitis

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1. Introduction

Multiple sclerosis is an inflammatory demyelinating disease of the central nervous system with pronounced neurodegeneration (Noseworthy et al., 2000; Trapp et al., 2004). It is the most common disabling neurological disease in young adults (Keegan and Noseworthy, 2002). The pathogenesis of multiple sclerosis is still unclear and the treatment is difficult. Therapies which reduce inflammation are available, but it is becoming increasingly clear that disability in multiple sclerosis is more highly correlated with neurodegeneration (Bjartmar et al., 2003). Up-regulatory triggers of neuroprotective pathways in the central nervous system are essential for the development of the next generation of the disease therapies (Azoulay et al., 2008).

Neurotrophins like brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are thought to play important roles in neuronal repair and plasticity (Boutros et al., 1997). Recent experimental evidence suggested neuroprotective effects of these proteins in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) have been proposed as therapeutic strategies in controlling EAE and multiple sclerosis disease (Arredondo et al., 2001; Makar et al., 2008). Furthermore, some of the approved therapies for multiple sclerosis such as interferon-β and glatiramer acetate could exert their beneficial effects by affecting these two neurotrophins in EAE mice (Azoulay et al., 2005; Boutros et al., 1997; Chen et al., 2003; Ziemssen et al., 2005). It has been reported that interferon-β is a potent promoter of NGF production by astrocytes and could induce BDNF production of peripheral blood mononuclear cells in multiple sclerosis patients (Azoulay et al., 2009; Boutros et al., 1997). Glatiramer acetate has been shown to induce BDNF secretion by glatiramer acetate-reactive T-helper cells lines (Chen et al., 2003; Ziemssen et al., 2005).

Minocycline is a second-generation, semi-synthetic tetracycline analog which has anti-inflammatory, immunomodulatory and neuroprotective activities. However, few studies have been carried out to assess its effects on the expression of neurotrophins in experimental autoimmune encephalomyelitis and multiple sclerosis (Azoulay et al., 2008). It has been shown minocycline could suppress disease activity and progression in EAE mice and multiple sclerosis patients and ameliorate the symptoms of EAE by inhibition of microglial activation, attenuation of apoptosis, suppression of free radical production, inhibition of matrix metalloproteinases, and regulation of leucocyte function (Chen et al., 2011; Yong et al., 2004). However, few studies have been carried out to assess minocycline's effects on the expression of neurotrophic factors in EAE mice or multiple sclerosis patients.

This study was to assess the effects of minocycline on the expression of BDNF and NGF in EAE mice. Our results demonstrated that minocycline could up-regulate the expression of BDNF and NGF in the sera, cerebral cortex, and lumbar spinal cord of EAE mice in vivo as well as the splenocytes culture supernatants in vitro. These suggest

previous paper
that up-regulation of neurotrophins in EAE may be a potential mechanism of minocycline in attenuating EAE in C57 BL/6 mice.

2. Materials and methods

2.1. Materials

Minocycline and complete Freund’s adjuvant were purchased from Sigma-Aldrich (St. Louis, MO). Myelin oligodendrocyte glycoprotein 35–55 (MOG 35–55) peptide (MEVGWYRPPFSRVQYRGK) was synthesized by CL-Bio Scientific CO., LTD. (Xi’an, China). Amino acid sequences were confirmed by amino acid analysis and mass spectroscopy. The purity of the peptide was greater than 95%. Mycobacterium tuberculosis H37RA was purchased from Difco (Detroit, MI). Pertussis toxin was purchased from Alexis Corp (San Diego, CA). RPMI 1640 and fetal calf serum was bought from Gibco/Life Technologies Inc. (Gaithersburg, MD). The cytokine assay by enzyme-linked immunosorbent assay (ELISA) kits for BDNF and NGF were purchased from Adlitteram Diagnostic Laboratories (Inc. USA). Polyclonal anti-BDNF and polyclonal anti-NGF were purchased from Santa Cruz.

2.2. Induction of experimental autoimmune encephalomyelitis

Six- to eight-week-old female C57 BL/6 mice weighing 16–18 g were obtained from Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Bioethics Committee of Sun Yat-sen University. Animals were housed with a 12-h light/dark cycle and at 22–23 °C and allowed free access to food and tap water. The induction of EAE was performed as described previously (Chen et al., 2009). Briefly, mice were immunized subcutaneously in the flanks with 200 μg of MOG 35–55 peptide per animal emulsified in complete Freund’s adjuvant containing 500 μg of M. tuberculosis H37RA. Immediately thereafter, and again 48 h later, the mice received an intraperitoneal injection of 300 ng of pertussis toxin in 100 μl of phosphate buffered saline (PBS). An additional injection of MOG35–55 peptide in complete Freund’s adjuvant was delivered 7 days later. The animals were examined and scored daily using the EAE rating scale. Disease was graded on 0–5 scale of increasing severity: 0, no signs; 1, loss of tail tonicity; 2, flaccid tail; 3, ataxia and/or paresis of hindlimbs; 4, complete paralysis of hindlimbs; 5, moribund or death.

2.3. Treatment of mice

Mice were randomly assigned to three groups: the control mice, the PBS-treated EAE mice, the minocycline-treated EAE mice. Each group has six animals. Minocycline was dissolved in PBS and administered at optimal dose to EAE mice: 50 mg/kg intraperitoneal injection, twice a day for the first 2 days, 50 mg/kg once daily for the next 5 days and 25 mg/kg once daily thereafter (Brundula et al., 2002; Chen et al., 2009). Treatment with minocycline was initiated at day 13 post immunization when the first mouse showed neurological symptoms and medication was administered daily until mice were sacrificed at day 40 post immunization. PBS-treated mice were administered with PBS only. Blood sera and splenocytes of different treated mice were prepared for neurotrophins assay by ELISA and the cerebral cortex and lumbar spinal cord from different treated mice were harvested for Weston blot at day 40 post immunization.

2.4. Histological analysis of demyelination and remyelination

Histological evaluation was performed on paraformaldehyde fixed, paraffin-embedded sections of lumbar spinal cords of EAE mice. Paraffin sections were stained with hematoxylin–eosin and solochrome cyanin impregnation for evaluating inflammatory infiltration and demyelination respectively. The scale evaluated for inflammation was as follows (O’Neill et al., 2006): 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, organization of inflammatory infiltrates around blood vessels; and 3, extensive perivascular cuffing with extension into adjacent parenchyma, or parenchymal infiltration without obvious cuffing. Demyelination in the spinal cords was scored as previously described (Kuerten et al., 2007): 1, traces of subpial demyelination; 2, marked subpial and perivascular demyelination; 3, confluent perivascular or subpial demyelination; 4, massive perivascular and subpial demyelination involving one half of the spinal cord with presence of cellular infiltrates in the central nervous system parenchyma; and 5, extensive perivascular and subpial demyelination involving the whole cord section with presence of cellular infiltrates in the central nervous system parenchyma.

2.5. ELISA assay

Sera and spleens of different treated mice were aseptically harvested. Splenocytes from three individual mice of each group were incubated in 96-well flat-bottom plates (1 × 10^6) in RPMI 1640 supplemented with 10% fetal calf serum, with the specific encephalitogenic peptide MOG 35–55 (15 μg/ml) used for the immunization. Culture supernatants were collected at 48 h. The production of BDNF and NGF in the sera and culture supernatants were determined by ELISA according to the manufacturer’s instructions. Neurotrophins concentrations were determined by interpolation from a standard curve by using the CurveExpert software.

2.6. Western blot analysis

Samples of the cerebral cortex and lumbar spinal cord from different treated mice were loaded on 12% gradient sodium dodecyl sulfate-polyacrylamide gels (80 μg protein per lane). Proteins were transferred onto nitrocellulose membrane (Bio-Rad). The membranes were incubated in a blocking buffer (20 mM Tris, 150 mM NaCl, pH 7.6, 0.1% Tween 20, TBST) and 5% non-fat milk. Afterward, the membranes were incubated with polyclonal anti-BDNF (1 μg/ml) and polyclonal anti-NGF (1 μg/ml) respectively. After 3 times washes with TBST buffer, the membrane was incubated with goat-anti-rabbit-HRP in the blocking buffer. The experiment was repeated three times. The production of BDNF and NGF in the sera and culture supernatants were determined by ELISA according to the manufacturer’s instructions. Neurotrophins concentrations were determined by interpolation from a standard curve by using the CurveExpert software.

2.7. Statistical analysis

Data are expressed as means ± S.E.M. and analyzed by SPSS 13.0 software. One-Way ANOVA and Mann–Whitney tests were used to determine the statistical significance. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Minocycline improved recovery of neurological function in EAE mice

In this study, we tested the effects of minocycline on EAE mice. The therapy was started at the day 13 post immunization when the first mouse showed neurological symptoms. The mean clinical scores of each group were shown in Fig. 1A. We observed that within the first 3 days of minocycline administration, the neurological deficits were stabilized, the maximum and average score for all animals in each group was significantly lower in the minocycline-treated EAE mice when compared with the PBS-treated EAE mice (P<0.01, Fig. 1A, B).
3.2. Minocycline reduced inflammation and demyelination of lumbar spinal cords in EAE mice

Because the most significant histopathological changes in animals with EAE were detected in the lumbar spinal cord (Eng et al., 1989), this portion of spinal cord was removed for analysis. Hematoxylin–eosin staining revealed an apparent infiltration of leukocytes into white matter of the lateral and anterior funiculus of lumbar spinal cord in the PBS-treated EAE mice, but this inflammatory infiltrates was significantly reduced by minocycline (Fig. 2A and B1,2), $P<0.01$. Next, the presence of demyelination was evaluated by solochrome cyanin technique. Corresponding with inflammatory infiltrates, minocycline significantly inhibited demyelination in the white matter of the lumbar spinal cord of EAE mice (Fig. 2A and B 3,4), $P<0.01$. The representative sections depicting the inflammatory infiltrates and demyelination in the lumbar spinal cord of EAE mice by minocycline or PBS administration were showed in Fig. 2B1-4.

3.3. Minocycline up-regulated BDNF and NGF production in the sera and splenocytes culture supernatants in EAE mice

To determine whether minocycline affect BDNF and NGF production, these two neurotrophins were assayed by ELISA in the sera and splenocytes culture supernatants of the different groups of mice. In the sera, the levels of BDNF and NGF were significantly lower in the PBS-treated EAE mice than in the control mice ($P<0.01$, Fig. 3A, B). However, the expression of these two neurotrophins in the minocycline-treated EAE mice was significantly higher than those of the control mice ($P<0.05$ for both BDNF and NGF, Fig. 3A, B) and the PBS-treated EAE mice ($P<0.01$ for both BDNF and NGF, Fig. 3A, B). In the splenocytes culture supernatants, the levels of BDNF and NGF were significantly lower in the PBS-treated EAE mice than in the control mice ($P<0.05$ for BDNF and $P<0.01$ for NGF, Fig. 3A, B). But in the minocycline-treated EAE mice, these two neurotrophins levels were much higher than those of the control mice ($P<0.05$ for BDNF and $P<0.01$ for NGF, Fig. 3A, B) and the PBS-treated EAE mice ($P<0.01$, Fig. 3A, B).
3.4. Minocycline up regulated the expression of BDNF and NGF in the cerebral cortex and lumbar spinal cord of EAE mice

We further investigated the expression of BDNF and NGF in the central nervous system (cerebral cortex and lumbar spinal cord) of different treated groups by Western blot. We found the BDNF expression was significantly lower in both the cerebral cortex and lumbar spinal cord of the PBS-treated EAE mice compared to the control mice (P<0.01, Fig. 4A). Minocycline could significantly up-regulate BDNF expression in these two regions of EAE mice than those of the control mice (P<0.01, Fig. 4A) and the PBS-treated EAE mice (P<0.01, Fig. 4A). When it comes to NGF, we found the similar results. NGF expression was significantly lower in the cerebral cortex and lumbar spinal cord of the PBS-treated EAE mice compared to the control group (P<0.05 for cerebral cortex and P<0.01 for lumbar spinal cord, Fig. 4B). Minocycline could significantly up-regulate NGF expression in these two regions of the EAE mice than those of the control mice (P<0.01, Fig. 4B) and the PBS-treated EAE mice (P<0.01, Fig. 4B).

4. Discussion

In this study, we confirmed that minocycline could significantly attenuate EAE in C57 BL/6 mice as our previously reported (Chen et al., 2009). The beneficial effects of minocycline on EAE may be related to multiple aspects, here we for the first time found that minocycline could up-regulate the expression of BDNF and NGF in the cerebral cortex and lumbar spinal cord as well as in the sera and culture supernatants of splenocytes in EAE C57 BL/6 mice.

BDNF and NGF are well-characterized neurotrophins involved in the differentiation and survival of a number of neurons localized in the peripheral and central nervous system (Aloe et al., 1994; Ebadi et al., 1997; Nomoto et al., 2007; Thoenen, 1997). These two neurotrophins undergo significant changes in the peripheral and central nervous system of multiple sclerosis patients and EAE animals (Caggiula et al., 2005; Sarchielli et al., 2002). Furthermore, it has been reported that the production of BDNF and NGF in central nervous system was strictly correlated with clinical outcome, and BDNF and NGF levels detected in patients with full recovery from relapse symptoms were significantly higher than that in patients with partial recovery (Caggiula et al., 2005; Laudiero et al., 1992; Sarchielli et al., 2002). The unavailability of BDNF and NGF from immune cells and loss of the neuroprotective effects of these cells may be related to more widespread phenomena of deviated immunity in multiple sclerosis, and may be linked to the continuous neuronal tissue loss in central nervous system during the course of this disease (Azoulay et al., 2008; Triaca et al., 2005). Thus, both BDNF and NGF were implicated in the pathogenesis of EAE and multiple sclerosis, and they have...
been proposed as therapeutic strategies in controlling the disease severity of EAE and multiple sclerosis (Arredondo et al., 2001; Makar et al., 2008).

In our study, we found BDNF and NGF levels were reduced in the sera of PBS-treated EAE mice. It has been reported that the transport of BDNF across the blood–brain barrier was negligible in normal conditions (Sakane and Pardridge, 1997). However, BDNF could be transported by the high-capacity unidirectional transport system through the inflamed blood–brain barrier in EAE because of its high permeability surface area product (De Santi et al., 2009, 2011; Pan et al., 1998b). And work on NGF has shown that it could be transported across the blood–brain barrier by the saturable transport systems in view of blood–brain barrier breakdown during EAE (Pan et al., 1998b). Moreover, BDNF and NGF could be imported from peripheral into the central nervous system by immune cells (Arredondo et al., 2001; Pan et al., 1998b). Reduced BDNF and NGF in sera of EAE mice also resulted in lower levels of these two neurotrophins in central nervous system. Minocycline could significantly up-regulate the production of BDNF and NGF in sera of EAE mice. The increased peripheral production of BDNF and NGF could result in an increased entry of these neurotrophins into the central nervous system (Arredondo et al., 2001; Ebadi et al., 1997; Kerschensteiner et al., 1999; Pan et al., 1998b). Furthermore, a significantly high production of BDNF and NGF by splenocytes of the minocycline-treated EAE mice was also detected in our experiment. These results suggested that minocycline could up-regulate the production of BDNF and NGF in peripheral system.

Besides in the sera and splenocytes culture supernatants, we also found that BDNF and NGF protein expression were reduced in the target organs (cerebral cortex and lumbar spinal cord) of the PBS-treated EAE mice, which was consistent with the previous studies and supported the notion that BDNF and NGF participate in the protective mechanisms against tissue damage in EAE (D’Intino et al., 2005; Kerschensteiner et al., 1999). The reduction of BDNF and NGF expression in central nervous system means the reduction of their protection or that there is an increase in the consumption of BDNF and NGF by the central nervous system due to the damaged tissue (D’Intino et al., 2005; Ebadi et al., 1997; Kerschensteiner et al., 1999). Minocycline could up-regulate the expression of BDNF and NGF in the brain and spinal cord of EAE mice. Given that BDNF and NGF play important roles in the protection or regeneration of brain tissue in EAE mice, up-regulation of these two neurotrophins expression by minocycline may help to slow down or even halt the disease severity of EAE, which is consistent with the previous studies reported by the high-capacity unidirectional transport system through the brain barrier by the saturable transport systems in view of blood–brain barrier breakdown during EAE (D’Intino et al., 2005; Ebadi et al., 1997; Kerschensteiner et al., 1999). Minocycline could up-regulate the production of BDNF and NGF in the brain and spinal cord of EAE mice. Given that BDNF and NGF play important roles in the protection or regeneration of brain tissue in EAE mice, up-regulation of these two neurotrophins expression by minocycline may help to slow down or even halt the disease severity of EAE, which is consistent with the previous studies reported by the high-capacity unidirectional transport system through the brain barrier by the saturable transport systems in view of blood–brain barrier breakdown during EAE (D’Intino et al., 2005; Ebadi et al., 1997; Kerschensteiner et al., 1999).

References


