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Regular Article

Angiopoietin-1 ameliorates inflammation-induced vascular leakage and improves functional impairment in a rat model of acute experimental autoimmune encephalomyelitis

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A B S T R A C T

Multiple sclerosis (MS) is characterized by perivascular inflammatory infiltration, secondary demyelination, and axonal loss in the central nervous system. Angiopoietin-1 (Ang-1) constitutes a family of endothelial growth factors that can inhibit MS-associated, inflammation-induced blood vascular leakage and lessen increased blood vessel permeability. This study was designed to investigate the effects of Ang-1 on a model of acute experimental autoimmune encephalomyelitis (EAE). Evans blue and the luciferase assay were employed to test blood vessel permeability, while immunohistochemistry, ELISA, and Western blotting were used to assess the degree of inflammation. Electron microscopy and cortical somatosensory evoked potentials were also used to observe axonal loss, white matter demyelination, and functional impairment in EAE groups. Our results showed that Ang-1 treatment could ameliorate inflammation-induced leakage, inhibit inflammatory cell infiltration into the brain and spinal cord, and improve functional impairment associated with EAE in a dose-dependent manner.

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Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by destruction of the blood–brain barrier (BBB), inflammatory cell infiltration, demyelination, axonal damage, and progressive paralysis (Hauser and Oksenberg, 2006). The activation of microglia, infiltration of lymphocytes, and extravasation of macrophages play important roles in disease development, whereas depletion of these inflammatory cells significantly decreases disease severity (Fang et al., 2013; Han et al., 2013). The BBB is a specialized structure composed of endothelial cells with tight junctions and astrocytes. Immune cells that have successfully passed through the BBB could destroy the microenvironment of the central nervous system (CNS); therefore, regulating immune cell passage over the BBB is essential for maintaining homeostasis in the healthy brain and for alleviating pathological changes in MS (Claudio et al., 1990). Previous research has indicated that macrophage depletion and microglial paralysis could significantly suppress the progress of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Rawji and Yong, 2013).

The fundamental stage in the onset of acute EAE is the breakdown of the BBB and rapid development of extensive edema and cellular inflammation (Fabis et al., 2007). Deterioration of the CNS microenvironment further causes demyelination, axonal degeneration, and clinical symptoms (Carson, 2002; Profyris et al., 2004). Angiopoietin-1 (Ang-1) belongs to a family of endothelial growth factors, which are well known for establishing and maintaining vascular maturation, stabilization, and integrity (Brindle et al., 2006; Nambu et al., 2004). Since Ang-1 has been shown to exert effects in inhibiting blood vessel leakage and reducing the infiltration of inflammatory cells (Han et al., 2010), we hypothesize that intravenous administration of Ang-1 would alleviate pathological changes and improve motor function associated with the EAE model.

Materials and methods

Animals and EAE induction

A total of 126 adult male Lewis rats were obtained from Zhejiang University Laboratory Animal Services Center. Of these, 14 were used as controls and the remaining 112 were randomly assigned into four groups (one vehicle-treated group and three Ang-1-treated groups,

Abbreviations: BBB, blood brain barrier; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; Ang-1, Angiopoietin-1; CNS, central nervous system; c-SEPs, cortical somatosensory-evoked potentials; LFB, Luxol fast blue; PBS, phosphate-buffered saline; EB, Evans blue; ELISA, enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon gamma; MBP, myelin basic protein; ANOVA, analysis of variance.

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n = 28/group). Experiments were carried out in accordance with NIH Guidelines for the Care and Use of Laboratory Animals, with approval from the animal ethics committee at the Zhejiang University.

EAE was induced in both Ang-1- and vehicle-treated rats, as previously described (Fang et al., 2013; Han et al., 2013). Beginning on day 7, animals were weighed and assessed for clinical signs of disease, the severity of which was assessed using a scale ranging from 0 to 5: grade 0 = no signs, grade 1 = partial loss of tail tonicity, grade 2 = total loss of tail tonicity, grade 3 = unsteady gait and mild paralysis, grade 4 = hind limb paralysis and incontinence, and grade 5 = moribund or death (Yu et al., 2010). The EAE model was generally considered a success if rats were assigned a score that exceeded 2. No animal scored above grade 4, and disease severity was assessed until the time of sacrifice.

Intravenous injection of Ang-1

The Ang-1 peptide (synthesized by Shanghai Science Peptide Biological Technology Co., Ltd, China) was dissolved in distilled water to a final concentration of 1 mg/ml. Since previous studies have shown Ang-1 to be more effective at 30, 100, and 300 μg/day compared to vehicle (Han et al., 2010), we chose a dose in the middle of the above-mentioned range (100 μg/day) as the minimum effective dose in our experiments; this dose has been shown to protect intestinal microvascular endothelial cells against radiation-induced death in adult mice (Cho et al., 2004). The first dose was given immediately after EAE induction, with access to the tail veins being achieved by beginning injections at the caudal end of the base of the tail and into one vein. Injection sites were moved to the alternate left or right side and became increasingly rostral on subsequent days. Solutions were intravenously injected each day for a period of 2 weeks.

Neurophysiological testing

Cortical somatosensory evoked potentials (c-SEPs) were recorded 2 (the peak stage of vehicle) and 8 weeks post immunization (the recovery stage of vehicle) for five rats in each group just before animals were sacrificed. Mice were fixed into a stereotaxic frame and the surgical processes were done according to previous experiments (Zhang et al., 2014). For registration of c-SEPs, screw electrodes were implanted over primary somatosensory cortical areas, and cerebellar reference electrodes were placed over the appropriate cortical area. SEPs were amplified, filtered, digitally converted, and stored for post-hoc analysis. Values obtained by the three series of stimulations were processed by statistical analyses. Peak positive and negative values were measured, and results were expressed as the mean ± SEM of voltage amplitude (μV) and latency (ms) (All et al., 2009; Devaux et al., 2003; Troncoso et al., 2000).

Perfusion and tissue processing

Animals in vehicle- and Ang-1-treated groups were sacrificed 2 and 8 weeks (5/time point in each group) post immunization. Brain cortical and spinal tissues were collected, and sections were prepared as previously described (Fang et al., 2011). A portion of each tissue was processed for histological assessment, immunohistological staining, and immunofluorescent staining. The remaining tissues were fixed in 2.5% glutaraldehyde solution and then examined by transmission electron microscopy.

Histology assessment

Cresyl violet (Nissl) staining was employed to assess inflammation and neuron survival counts. Neuron counts were restricted to cells with a well-defined nucleolus and a cell body that displayed adequate amounts of endoplasmic reticulum. Digital images were collected using a Nikon TE-300 microscope in five sections/animal and three visual fields/section under 200× magnification bright-field viewing. An assessment of the severity of inflammatory cell infiltration was scaled as follows (Ma et al., 2010): 0, no inflammation; 1, cellular infiltrates only around blood vessels and meninges; 2, mild cellular infiltrates in parenchyma (1–10/section); 3, moderate cellular infiltrates in parenchyma (11–100/section); and 4, serious cellular infiltrates in parenchyma (100/section).

Luxol fast blue (LFB) staining was used to evaluate the degree of demyelination, as previously described (Fang et al., 2013). Digital photomicrographs were obtained at 40× magnification, and the amount of demyelination was scored as follows (Yin et al., 2010): 0, normal white matter; 1, rare foci; 2, a few areas of 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 CNS parenchyma; and 5, extensive perivascular and subpial demyelination involving the whole cord section with presence of cellular infiltrates in the CNS parenchyma.

Bielschowsky silver staining was performed to estimate axonal loss (as previously described by Fang et al., 2013; Han et al., 2013), which was assessed using the following scale (Yin et al., 2010): 0, no loss; 1, a few foci of superficial loss involving less than 25% of tissue; 2, foci of deep axonal loss, encompassing over 25% of tissue; and 3, diffuse and widespread axonal loss.

Luciferase assay

To quantify the permeability of the blood–spinal barrier to proteins, rats (n = 3/group) at 2 and 8 weeks post immunization were injected with 80 ml of a 0.5 mg/ml solution of luciferase (L9506; Sigma) in 0.05 M phosphate-buffered saline (PBS)/0.001% bovine serum albumin into the jugular vein 30 min before processing (Whetstone et al., 2003). After flushing out blood by PBS perfusion, a 5-mm block (epicenter 2.5 mm) was collected and protein was immediately extracted from the tissue in lysis buffer (E1500; Promega). The sample was then centrifuged and the supernatant was used to measure enzyme activity with a luciferase assay kit (E1500; Promega) and a luminometer. All samples were analyzed in triplicate.

Assessment of BBB disruption with Evans blue extravasation

At 2 and 8 weeks post immunization, rats (n = 3/group) were randomly selected for assessment of BBB vascular permeability with a modified Evans blue extravasation method. Briefly, at 2 and 8 weeks post immunization, rats were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal injection) and infused with 37 °C Evans blue dye (2% in 0.9% normal saline, 4 ml/kg) via the right femoral vein over 5 min. Two hours later, rats were perfused with 300 ml normal saline to wash out any remaining dye in the blood vessels and then brains and spinal cord tissue were removed and sectioned to 2 mm in thickness. BBB permeability was then evaluated in the brain motor cortex and spinal cord tissue. Absorbance of Evans blue in the supernatant was then measured with a spectrophotometer (Molecular Devices OptiMax, USA) at 610 nm. Dye concentrations were expressed as μg/g of tissue weight and calculated from a standard curve obtained from known amounts of the dye.

Immunohistochemical staining

Immunohistochemical staining of anti-CD4 (1:500, AbCam, Cambridge, MA), anti-tumor necrosis factor alpha (TNF-α 1:1000, ProSci Incorporated, CA, USA), anti-CD45 (1:500; AbCam, Cambridge, MA), and mouse anti-myelin basic protein (MBP, 1:500, AbCam, Cambridge, MA) was performed as previously described (Fang et al., 2013; Han et al., 2013). Five sections from the motor cortex and anterior horns of the spinal cord of each animal were randomly selected and

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