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Soluble CD40 ligand disrupts the blood–brain barrier and exacerbates inflammation in experimental autoimmune encephalomyelitis

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ABSTRACT

Serum soluble CD40 ligand (sCD40L) has been reported to positively correlate with the albumin quotient, a marker of blood–brain barrier (BBB) breakdown, in patients with multiple sclerosis (MS). To clarify the mechanisms of sCD40L in MS pathophysiology, sCD40L was administered to experimental autoimmune encephalomyelitis (EAE) mice and a human brain microvascular endothelial cell (HBMEC)-based BBB model. The high-dose sCD40L group showed a worse EAE score than the low-dose and control groups. BBB permeability was increased by administering sCD40L in a HBMEC-based BBB model. Thus, sCD40L induces more severe inflammation in the central nervous system by disrupting the BBB.

1. Introduction

Multiple sclerosis (MS) is a demyelinating and neurodegenerative disease of the central nervous system (CNS). A feature of MS is dissemination in time and space. Although the etiology of MS remains unclear, both T and B cells have been reported to play important roles in the pathogenesis of MS (Hartung et al., 2014). Moreover, blood–brain barrier (BBB) breakdown has been recognized as a critical step in MS pathology (Absinta et al., 2015; Spencer et al., 2018). Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS in which T cells expressing interleukin-17 (IL-17) infiltrate the CNS at the early stage of EAE. Therefore, T helper 17 (Th17) cells are considered a key factor in the pathogenesis of EAE (Kurschus, 2015).

CD40 ligand (CD40L) is a transmembrane protein that plays an important role in the immune system. The main function of CD40L is to produce high-affinity B cells (Elgueta et al., 2009). Recent studies have suggested that CD40L increases the permeability of the BBB (Davidson et al., 2012; Ishikawa et al., 2005). Soluble CD40L (sCD40L) has been reported to have the same function as CD40L (Vakkalanka et al., 1999). In the peripheral blood, sCD40L has been found to be produced only by shedding from the surface of activated platelets or activated T cells and exists in trimers (Aloui et al., 2014). The molecular weight of sCD40L is 18 kDa, which is much smaller than albumin (66 kDa) (Becker, 2004; Pietravalle et al., 1996). CD40L works by binding to CD40, which is expressed on the surface of B cells and other cells, including macrophages, dendritic cells, smooth muscle cells, microglia, astrocytes, and endothelial cells. Astrocytes and endothelial cells are also the components of the BBB.

Previously, we reported elevated serum sCD40L levels in patients with MS and a positive correlation between serum sCD40L levels and the albumin quotient (Qalb) (Masuda et al., 2017). Qalb gives the cerebrospinal fluid (CSF)/serum albumin ratio, which is a marker of BBB dysfunction. Meanwhile, CD40–CD40L interaction has been reported to be critical for Th17 differentiation (Iezzi et al., 2009). We hypothesized that sCD40L could cause BBB breakdown or severe inflammation through the upregulation of Th17 cells in MS pathogenesis. Therefore, we investigated the function of sCD40L in EAE mice and an in vitro study using human brain microvascular endothelial cells (HBMEC).

Abbreviations: BBB, blood–brain barrier; EAE, experimental autoimmune encephalomyelitis; CD40L, CD40 ligand; CNS, central nervous system; HAND, HIV-associated neurocognitive disorder; HBMEC, human brain microvascular endothelial cells; IL-6, interleukin-6; IQR, interquartile range; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NMO, neuromyelitis optica; NMOSD, neuromyelitis optica spectrum disorder; Qalb, albumin quotient (CSF/serum albumin ratio); sCD40L, soluble CD40L

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2. Materials and methods

2.1. EAE induction in mice

Ten-week-old C57BL/6 female mice (18–25 g) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed in specific pathogen-free facilities at Chiba University with a maximum of four animals per cage, with free access to water and standard rodent chow. EAE was induced using immunization with myelin oligodendrocyte glycoprotein (MOG). A total of 200 mg MOG peptide 35–55 in complete Freund's adjuvant containing 400 mg of killed *Mycobacterium tuberculosis* H37Ra was injected subcutaneously at two sites. On the days of immunization (days 0 and 1), 200 ng pertussis toxin was injected intraperitoneally using Hooke kits (EK-0105; Hooke Laboratories, Lawrence, MA, USA). EAE was scored on the following scale: 0 = no clinical signs; 1 = partial paralysis of tail; 2 = flaccid tail; 3 = limp tail and partial weakness of hind legs; 4 = limp tail and complete weakness of hind legs; 5 = limp tail, complete weakness of hind legs and partial weakness of front legs; and 6 = complete hind and front legs paralysis. The intermediate condition of each score was assessed as 0.5. Dr. Uzawa immunized the mice, and EAE clinical scores were assessed blindly by Dr. Masuda to avoid subjective bias.

2.2. Treatment with sCD40L

Recombinant mouse sCD40L was obtained from Abnova (P4590; Taipei, Taiwan). Mice were injected intraperitoneally on days 0 and 1 with recombinant mouse sCD40L resuspended in phosphate buffered saline with 0.1% bovine serum albumin (BSA) at the same time with Pertusis Toxin (PT) administration. Injection side of sCD40L was opposite from the injection side of PT. EAE mice were divided into three groups, the high sCD40L group (0.2 mg/g body weight), the low sCD40L group (0.05 mg/g body weight), and the control group (only BSA was administered). Each group comprised 12 mice. The concentration of sCD40L was determined based on previous research (Davidson et al., 2012).

2.3. Serum Th17 cytokine assays in EAE mice

To assess sCD40L affecting Th17 cells in the early phase of EAE, we measured serum Th17 cytokines including IL-17E, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, macrophage inflammatory protein (MIP-3a), IL-1b, IL-2, IL-4, IL-5, IL-6, IL-21, IL-22, IL-28B, IL-10, IL-23, IL-12p70, IL-27, IL-13, IL-15, IL-17A, IL-17F, IL-33, IL-31, tumor necrosis factor (TNF)β, TNFα, and CD40L on day 14 (at the early phase of the peak). All serum samples were centrifuged at 3000 rpm for 10 min. Just after centrifugation all serum samples were immediately stored at − 80 °C until analysis. Cytokine measurements were performed using a multiplexed fluorescent magnetic bead-based immunoassay (Merck Millipore, Darmstadt, Germany), according to the manufacturer's instructions. Cytokine levels were calculated with reference to a standard curve for each cytokine.

2.4. BBB permeability assay

Recombinant human sCD40L was obtained from Enzo Life Sciences (ALX-522-015-2010; Farmingdale, NY, USA). We performed sodium fluorescein (Na-F) permeability assay using the HBMEC/ci18-based in vitro BBB method (Kitamura et al., 2017). Ci18 cell line is a sister clone of HBMEC/ciβ, and the assay was performed as reported previously (Kamiichi et al., 2012). Briefly, HBMEC/ci18 were seeded at 4.0×10^5 cells/mL on a membrane filter of an insert culture system (polyethylene terephthalate, 0.4-mm high-density pores, and 0.3 cm^2, BD Falcon, Franklin Lakes, NJ, USA), and then cultured for 3 days. Before the assay, the cells were pre-incubated over 30 min at 37 °C with Ringer-Hepes buffer (136 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10 mM NaH_2PO_4, 25 mM glucose, and 10 mM Hepes; pH 7.4). Twenty-four hours before the initiation of the assay, human sCD40L was administered to the insert (apical side). The recombinant human sCD40L was resuspended in sterile water with 0.5% BSA. The concentration of sCD40L was 100 ng/mL based on the previous research (Ramirez et al., 2010). The assay was initiated by adding Na-F (Sigma) (150 ng/insert) to the insert at 37 °C. Immediately after the initiation (0 min) and after incubation for 30, 60, and 90 min, the medium was collected from the well (basolateral side). The fluorescence of Na-F in each medium was determined using an ARVO-SX (PerkinElmer, Waltham, MA, USA) with wavelengths ex/em¼485 nm/535 nm. The permeability coefficients (Pe, cm/min) were calculated. The experiment was performed five times.

2.5. Statistical analysis

Continuous data were compared using the Mann-Whitney *U* test with a Bonferroni correction. To consider the multiple testing problem, we applied the Bonferroni correction on the computed *P* values to reduce type I errors. A *P* value of < 0.05 was considered statistically significant. Statistical tests were conducted using SPSS version 24.0 (IBM Corporation, Armonk, NY, USA).

2.6. Ethics

The study procedure in animal experiments was approved by the ethics committee of Chiba University School of Medicine (No. A27-209).

3. Results

3.1. EAE severity was increased by sCD40L administration

The result of EAE study is shown in Fig. 1. The result showed the high sCD40L group exacerbated EAE clinical score compared with the other two groups. The high sCD40L group showed the worse clinical score on days 18, 21–25 ($P < 0.05$), and days 23 and 26 ($P < 0.01$) compared with the control group. Meanwhile, the result showed a

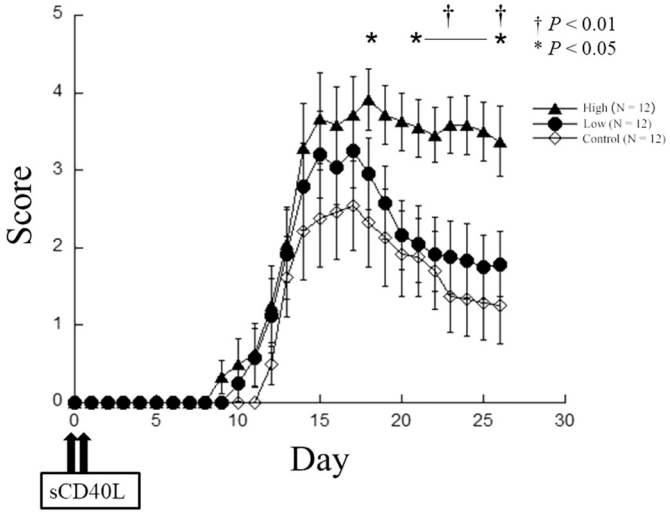

Fig. 1. Effects of sCD40L administration to EAE mice.
The straight line shows the high sCD40L EAE group (0.2 mg/g body weight of sCD40L and BSA). The dotted line shows the low sCD40L EAE group (0.05 mg/g body weight of sCD40L and BSA). The long-short dashed line shows the control EAE group (BSA without sCD40L). Compared with the control EAE group, the high sCD40L EAE group showed higher clinical EAE scores on days 18, 21–25 ($P < 0.05$) and days 23 and 26 ($P < 0.01$). Results are mean ± standard error of the mean. *$P < 0.05$ and †$P < 0.01$ by Mann-Whitney *U* test between the high sCD40L EAE group and the control EAE group. EAE, experimental autoimmune encephalomyelitis; sCD40L, soluble CD40 ligand.