Blockade of Glutamate Release from Microglia Attenuates Experimental Autoimmune Encephalomyelitis in Mice

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Multiple sclerosis (MS) is a chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system. Despite a variety of anti-inflammatory or immunomodulation drugs including interferon-β are effective to reduce relapse risk, most patients have progressive neurological deterioration due to axonal degeneration. Accumulation of activated microglia is a pathological hallmark of active MS lesion. Microglia can act as not only antigen-presenting cells but also effector cells to damage other cells in the central nervous system. Especially, glutamate released by activated microglia induces excitotoxicity and may contribute to neurodegeneration in MS. Gap junction is a major cell-to-cell channel and is composed of paired hemichannels on coupled cells. Recent studies showed that cells release various small molecules (including ions, ATP, and amino acids) from unpaired hemichannel of gap junction that is openly exposed to the extracellular space. We have previously revealed that activated microglia produce glutamate via glutaminase and release it through hemichannels of gap junctions. Thus, in this study, we examined whether the glutaminase inhibitor and the gap junction blocker relieved experimental autoimmune encephalomyelitis (EAE) that is an animal model of MS. Here we show that the gap junction blocker carbenoxolone (CBX) and the glutaminase inhibitor 6-diaz-5-oxo-L-norleucine (DON) decreased glutamate release from activated microglia and rescued neuronal death in a dose-dependent manner in vitro. In EAE mice, treatment with CBX or DON also attenuated EAE clinical symptoms. Thus, blockade of glutamate release from activated microglia with CBX or DON may be an effective therapeutic strategy against neurodegeneration in MS. ——— microglia; excitotoxicity; gap junction; glutaminase; multiple sclerosis.

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have also demonstrated that both the glutaminase inhibitor and the gap junction blocker prevented neuronal death in a rodent model of transient ischemic brain injury (Takeuchi et al. 2008). Thus, here we examined whether the glutaminase inhibitor or the gap junction blocker ameliorated experimental autoimmune encephalomyelitis (EAE), an animal model of MS.

METHODS

Animals and reagents

All protocols were approved by the Animal Experiment Committee of Nagoya University. C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). All reagents except those otherwise specified were obtained from Sigma (St. Louis, MO, USA).

Cell culture

Microglia were prepared from newborn C57BL/6J mice with the ‘shaking off’ method as described previously (Suzumura et al. 1987; Takeuchi et al. 2005). Microglia were cultured in 24-well dishes at a density of 5 × 10^4 cells/well. The purity of the cultures was more than 99% as determined by CD11b-specific immunostaining.

Neuronal cultures were prepared from C57BL/6J mice at embryonic day 17 as described previously (Takeuchi et al. 2005; Takeuchi et al. 2006). Briefly, cortices were dissected and freed of meninges. Cortical fragments were dissociated into single cells using dissociation solution, and they were resuspended in Neuron Medium (Sumitomo Bakelite, Akitai, Japan). Primary neuronal cells were plated on 12 mm-poly-ethyleneimine-coated cover slips (Asahi Techno Glass Corporation, Chiba, Japan) in 24-well multidiishes at a density of 5 × 10^4 cells/well. The purity of the cultures was more than 95% as determined by NeuN-specific immunostaining. Activated microglia treated with 1 μg/ml lipopolysaccharide (LPS) were incubated with each drug at the following concentrations: glutaminase inhibitor, 0.1, 0.5, and 1 mM 6-diazo-5-oxo-L-norleucine (DON); gap junction inhibitor, 10, 50, and 100 μM carbenoxolone disodium (CBX). After a 24-h incubation, microglia conditioned medium was applied to each well containing 5 × 10^4 neurons at 10-13 days in vitro. Evaluations of neuronal damage were performed 24 h after medium exchange. Cell death was evaluated by dye-exclusion with propidium iodide (Molecular Probes, Eugene, OR, USA) as described previously (Takeuchi et al. 2005; Takeuchi et al. 2006). To measure extracellular glutamate concentrations, we used the Glutamate Assay Kit colorimetric assay (Yamas Corporation, Tokyo, Japan) as described previously (Takeuchi et al. 2005; Takeuchi et al. 2006). Each assay was carried out in six independent trials. Neurons were fixed with 4% paraformaldehyde overnight and cryoprotected in 10–30% sucrose at 4°C. Then, they were embedded in O.C.T. compound (Tissue Tek, Elkhart, IN, USA) and frozen in liquid nitrogen. Sections (8 μm) were stained with hema-toxylin and eosin. Stained sections were observed with a microscope (BZ-8000, Keyence).

Statistical analysis

All results were analyzed by one-way or two-way analysis of variance (ANOVA) with a Tukey-Kramer post-hoc test using GraphPad Prism version 5.0 (GraphPad Software, Inc. La Jolla, CA, USA).

RESULTS

Blockade of microglial glutamate release rescued neuronal death.

We previously demonstrated that activated microglia/macrophage synthesized glutamate by glutaminase from extracellular glutamine and released it through hemichannels of gap junctions (Takeuchi et al. 2006; Yawata et al. 2008). We also revealed that glutamate from activated microglia/macrophage induced excitotoxic neuronal death through NMDA receptor signaling (Takeuchi et al. 2006; Yawata et al. 2008). Thus, we assessed whether inhibition of gap junction or glutaminase reduced microglial excitotoxicity. LPS-stimulated microglia released large amounts of glutamate and induced severe neuronal death in vitro (Fig. 1B, 1E and 1F). In contrast, administration of

Active induction of EAE

EAE mice were produced as described previously (Kato et al. 2004; Sonobe et al. 2007; Wang et al. 2008). Briefly, C57BL/6J mice aged 6-8 weeks were immunized subcutaneously at the base of the tail with 0.2 ml of emulsion containing 200 μg MOG_{35-55} peptide (MEGVYRSPFSRVHYRNGK, Operon Biotechnologies, Tokyo, Japan) in saline combined with an equal volume of incomplete Freund’s adjuvant containing 300 μg heat-killed Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI, USA). Mice were injected with 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA, USA) intraperitoneally on the day of immunization and two days after immunization. CBX or DON was administered intraperitoneally every other day from the day of immunization at each dosage (CBX: 0.2, 2, or 20 mg/kg; DON: 0.016, 0.16, or 1.6 mg/kg). Control animals were injected with the equal volume of vehicle, phosphate-buffered saline (PBS). Mice were assessed daily for clinical signs of EAE according to the following scale designated as EAE clinical score: 0 - normal; 1 - limp tail or mild hind limb weakness; 2 - moderate hind limb weakness or mild ataxia; 3 - moderate to severe hind limb weakness; 4 - severe hind limb weakness, mild forelimb weakness, or moderate ataxia; 5 - paraplegia with moderate forelimb weakness; 6 - paraplegia with severe forelimb weakness, severe ataxia, or moribundity. Control mice developed peak EAE score (4-5) approximately 14-20 days after immunization as described previously (Kato et al. 2004; Sonobe et al. 2007; Wang et al. 2008). The first day when mice showed overt EAE clinical sign (a score 1) was determined as EAE onset day. Duration of severe EAE (a score 4) was assessed as severe EAE days. The most severe clinical score during EAE was evaluated as peak clinical score. We continued drug administration and followed clinical evaluation for five weeks after immunization.

Histological analysis

Mice with peak EAE were anaesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M PBS. Lumbosacral spinal cords were immediately removed, postfixed in 4% paraformaldehyde overnight, and cryoprotected in 10–30% sucrose at 4°C. Then, they were embedded in O.C.T. compound (Tissue Tek, Elkhart, IN, USA) and frozen in liquid nitrogen. Sections (8 μm) were stained with hema-toxylin and eosin. Stained sections were observed with a microscope (BZ-8000, Keyence).

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Both CBX and DON prevent neuronal death via reduction of microglial glutamate release in a dose-dependent manner. (A–D) Images of MAP-2-stained cortical neuronal cultures 24 h after treatment with microglial conditioned media. (A) Neurons with non-treated microglial conditioned media. (B) Neurons with LPS-treated microglial conditioned media. (C) Neurons with LPS- and 100 μM CBX-treated microglial conditioned media. (D) Neurons with LPS- and 1 mM DON-treated microglial conditioned media. Scale bar, 20 μm. (E) Extracellular glutamate concentration. (F) Ratio of neuronal death. *, p < 0.05 vs neurons with LPS-treated microglial conditioned media. †, p < 0.05 vs neurons with LPS- and 10 μM CBX- or 0.1 mM DON-treated microglial conditioned media. §, p < 0.05 vs neurons with LPS- and 50 μM CBX- or 0.5 mM DON-treated microglial conditioned media. Data represent the means ± s.d. of six independent trials.
the gap junction blocker CBX or the glutaminase inhibitor DON prevented LPS-induced microglial glutamate release and subsequent neuronal death in a dose-dependent manner (Fig. 1C-1F). These drugs did not suppress glutamate production in control neuronal cultures (data not shown).

**Treatment with the gap junction blocker or the glutaminase inhibitor attenuated EAE.**

Next, we evaluated whether inhibition of gap junction or glutaminase ameliorated EAE. Although all mice treated with drugs developed EAE, administration of 2 mg/kg CBX
or 1.6 mg/kg DON significantly diminished the clinical score of EAE (Fig. 2). Treatment with 2 mg/kg CBX significantly delayed EAE onset day, shortened the duration of severe EAE, and attenuated peak EAE clinical score. Treatment with 1.6 mg/kg DON also significantly delayed EAE onset day and shortened the duration of severe EAE, but did not alter peak EAE clinical score compared to vehicle-treatment. The dose effect of CBX was bell-shaped whereas DON ameliorated EAE at the maximum dosage (Fig. 2C-E). Histological analysis of the lumbar spinal cord revealed that treatment with 2 mg/kg CBX diminished inflammatory cell infiltration, whereas treatment with 1.6 mg/kg DON did not (Fig. 2F-H). These histological findings agreed with the clinical data because CBX attenuated peak EAE clinical score, while DON did not.

**Discussion**

At present, the main therapeutic strategy against MS is to soothe autoimmunity by anti-inflammatory drugs or immunomodulation drugs (Coyle 2007). However, the novel therapies are needed that focus on prevention of neurodegeneration because most patients are suffered from progressive axonal degeneration due to excitotoxicity (Trapp et al. 1998; Dutta and Trapp 2007). One approach is the blockade of glutamate receptors on neurons (Pitt et al. 2000), however, adverse effects increase in a dose-dependent manner due to perturbing the physiological glutamate signaling. Another approach is the blockade of microglia/macrophage glutamate release. We have previously identified that TNF-α is a key cytokine that induces microglial glutamate release by upregulating glutaminase and gap junctions (Takeuchi et al. 2006). Treatment with TNF-α neutralizing antibodies is an effective therapy for autoimmune diseases, however, this therapy has serious adverse side effects including an increased risk for infections and cancer. While cellular homeostasis of glutamate levels may be maintained primarily by the glutamate dehydrogenase pathway (Nissim 1999), activated microglia/macrophage produce glutamate through a separate pathway involving the enzyme glutaminase and gap junctions (Takeuchi et al. 2006; Yawata et al. 2008). Thus, we here propose the gap junction blocker CBX and the glutaminase inhibitor DON as promising therapeutic agents that may not perturb glutamate homeostasis. Importantly, these drugs have been used clinically for other purposes (CBX, anti-inflammatory drug; DON, antitumor drug). In the present study, we demonstrated that CBX and DON decreased glutamate release from activated microglia and subsequent neuronal death in a dose-dependent manner *in vitro*. Moreover, we also revealed that administration of CBX or DON ameliorated clinical symptoms of EAE mice, while the effective dose of these drugs was relative limited. Blockade of gap junction may exert neuroprotection additively because neuronal and astrocytic gap junctions were also considered to contribute to neuronal damage (Chanson et al. 2005), which may lead to the difference of efficacy between CBX and DON. We previously confirmed that IFN-β also attenuated microglial glutamate release by downregulation of glutaminase (Jin et al. 2007). Thus, the combined therapy of IFN-β and CBX or DON may be the novel strategy against MS, while we need further investigations to determine the optimal dosage.

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