Copper/zinc chelation by clioquinol reduces spinal cord white matter damage and behavioral deficits in a murine MOG-induced multiple sclerosis model

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A R T I C L E   I N F O

Article history:
Received 21 August 2012
Revised 19 December 2012
Accepted 17 January 2013
Available online 27 January 2013

Keywords:
Experimental autoimmune encephalomyelitis
Multiple sclerosis
Copper
Zinc
Microglia
BBB disruption
MMP-9
Autophagy

A B S T R A C T

The present study aimed to evaluate the therapeutic potential of clioquinol (CQ), a metal chelator, on multiple sclerosis pathogenesis. Experimental autoimmune encephalomyelitis was induced by immunization with myelin oligodendrocyte glycoprotein (MOG(35–55)) in female mice. Three weeks after the initial immunization, demyelination and immune cell infiltration in the spinal cord were analyzed. CQ (30 mg/kg) was given by gavage once per day for the entire experimental course. CQ profoundly reduced the daily clinical score and incidence rate of EAE mice. The CQ-mediated inhibition of the clinical course of EAE was accompanied by suppression of demyelination and reduced infiltration by encephalitogenic immune cells including CD4, CD8, CD20 and F4/80 positive cells. CQ also remarkably inhibited EAE-associated BBB disruption and MMP-9 activation. Autophagy contributes to clearance of aggregated proteins in astrocytes and neurons. The present study found that EAE increased the induction of autophagy and CQ further increased this expression. Furthermore, the present study found that post-treatment with CQ also reduced the clinical score of EAE and spinal cord demyelination. These results demonstrate that CQ inhibits the clinical features and neuropathological changes associated with EAE. The present study suggests that transition metals may be involved in several steps of multiple sclerosis pathogenesis.

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Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS). MS is associated with inflammation in the CNS and destruction of the myelin sheath that surrounds the axon of neurons. Several lines of evidence suggest that MS is an autoimmune disorder characterized by the generation of T cells that are directed against endogenous, host-derived myelin proteins; this inappropriate self-targeting is thought to drive the neuropathology and clinical development of this disease (Trapp and Nave, 2008).

Among the several types of neurodegenerative diseases in which inflammatory processes are involved, MS is one that is induced by a dysfunctional immune response. Peripheral T cells that are autoreactive for myelin migrate into the CNS and initiate cytotoxic processes that include demyelination and axonal degeneration. Demyelination leads to neurological deficits such as sensory disturbances, motor weakness, and progressive disability (Compston and Coles, 2008). Infiltrating T cells are constantly reactivated within the CNS parenchyma by microglia. Cytokine release and constant antigen presentation by microglia potentiates T-cell recruitment to the CNS and facilitates their polarization into cytotoxic phenotypes. Inhibition of their function can attenuate disease progression in experimental animal models of MS supporting their role in initiation and development of this disease (Heppner et al., 2005; Huitinga et al., 1990; Kozela et al., 2011). Thus, suppression of microglia may reduce inflammatory chain reactions and limit demyelination in MS.

The spinal cord is a specialized region of the central nervous system that contains concentrated metal ions including zinc, copper and iron in the synaptic terminals of its neurons. Among these metal ions, zinc is packaged into synaptic vesicles by zinc transporter 3 (ZnT3) and released during synaptic activity from excitatory synaptic terminals. This zinc release has been suggested to contribute to neuronal death in several acute disease conditions, such as seizures (Frederickson et al., 1988), ischemia (Tonder et al., 1990), traumatic brain injury (Suh et al., 2000a) and hypoglycemia (Suh et al., 2004; Suh et al., 2007). Brain zinc accumulation is also a prominent feature of advanced Alzheimer disease (AD) and is biochemically linked to brain amyloid beta-peptide accumulation and dementia severity in AD (Bush et al., 1994; Cherny et al., 2001;
Suh et al., 2000b). A recent study showed that cell death induced in mitochondrial inhibitor models of Huntington’s and Parkinson’s disease was induced by zinc accumulation, which was attenuated by inhibition of zinc neurotoxicity (Sheline et al., 2013). Copper levels are also high and this ion may also be released into the synaptic space by neuronal activity (Hartter and Barnea, 1988; Kardos et al., 1989). Secreted copper modulates neuronal activity by several channels, such as N-methyl-D-aspartate (NMDA), gamma-Aminobutyric acid (GABA), and glycine receptors (Kardos et al., 1989; Trombley and Shepherd, 1996). Although the pathological roles of transition metals in neurodegenerative disease have been investigated, the role of endogenous transition metals in MS has not yet been well-characterized. Altered serum or plasma concentrations of copper and zinc have been reported in multiple sclerosis (Kapaki et al., 1989; Melo et al., 2003).

Clioquinol (5-chloro-7-iodo-8-hydroxy-quinoline, CQ) is a metal chelator. Recent animal studies have shown that CQ can reverse the progression of Alzheimer’s, Parkinson’s and Huntington’s diseases (Cherny et al., 2001; Kaur et al., 2003; Nguyen et al., 2005). Evidence from clinical trials suggests that CQ may halt cognitive decline in Alzheimer’s disease, possibly owing to its ability to chelate copper and zinc ions. CQ treatment of transgenic Huntington’s mice decreased Huntington aggregate accumulation, decreased striatal atrophy, improved behavioral performance, and extended lifespan (Nguyen et al., 2005). Recent studies have demonstrated that CQ not only acts as an extracellular metal chelator but also as a zinc ionophore under physiological conditions (Bush, 2008). Park et al. presented an interesting study that CQ induces autophagy in a zinc-dependent manner and contributes to clearance of aggregated proteins in astrocytes and neurons. Hence, in addition to its metal-chelating effect in and around amyloid beta (Aβ) plaques, CQ may contribute to the reduction of Aβ loads by inducing autophagy and/or by normalizing extracellular/intracellular zinc levels in the brain (Park et al., 2011).

One of the best described and most commonly used animal models of MS is experimental autoimmune encephalomyelitis (EAE), which is induced when animals are immunized with myelin oligodendrocyte glycoprotein (MOG) (Mendel et al., 1995; Shevach et al., 1999). Using the MOG-induced EAE mouse model, we investigated if systemically delivered CQ could suppress disease progression. In the present study, CQ ameliorated the severity of the EAE in MOG-injected mice, which is accompanied with reduction of demyelination, attenuation of microglia activation, inhibition of proliferation of and infiltration by encephalitogenic immune cells, reduction of MMP-9 activation and augmentation of autophagy induction. These data establish transition metal chelation as a potential therapeutic strategy in MS.

Materials and methods

Induction of EAE

Animal use and relevant experimental procedures were approved by the Institutional Animal Care and Use Committee, Hallym University (Protocol # Hallym 2011-68). This manuscript was written up in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines (Kilkenny et al., 2012). C57BL/6 female mice, aged 8 weeks, were purchased from DBL (Chungcheongbukdo, Korea), were housed in a temperature- and humidity-controlled environment, and supplied with Purina diet (Purina, Gyeonggido, Korea) and water ad libitum. Mice were immunized on day 0 by subcutaneous injection of 200 μl of a mixture of recombinant myelin oligodendrocyte glycoprotein (MOG(35–55)) (AnaSpec, CA) and complete Freund’s adjuvant containing 400 μg of Mycobacterium tuberculosis H37Ra (Difco Laboratories, MI) according to the manufacturer’s instruction. Pertussis toxin (List Biological Laboratories, CA) was intraperitoneally administered at a dose of 400 ng on post-immunization days 0 and 2. A booster injection was given on day 7 after the initial immunization.

Clioquinol administration

CQ (CQ, Sigma, St Louis, MO) was dissolved with DMSO and diluted with saline 10 times (10% DMSO). CQ was delivered by gavage once per day at a dose of 30 mg/kg/day from day 0 until the end of the experiment. Animals were divided into four groups: (1) sham without CQ (DMSO only, n=15), (2) sham with CQ (CQ only, n=14), (3) MOG without CQ (MOG + DMSO, n=21), (4) MOG with CQ (MOG+CQ, n=19). To test the post-treatment effects of CQ, CQ was delivered three days after the typical onset of clinical symptom (15 days after first MOG injection).

Behavioral testing

Behavior was scored daily for evaluation of clinical features of EAE according to the following criteria: 0, no deficit; 0.5, partial loss of tail tone or slightly abnormal gait; 1.0, complete tail paralysis or both partial loss of tail tone and mild hind limb weakness; 1.5, complete tail paralysis and mild hind limb weakness; 2.0, tail paralysis with moderate hind limb weakness (evidenced by frequent foot dropping between bars of cage top while walking); 2.5, no weight-bearing on hind limbs (dragging) but with some leg movement; 3.0, complete hind limb paralysis with no residual movement; 3.5, hind limb paralysis with mild weakness in forelimbs; 4.0, complete quadriplegia but with some movement of head; 4.5, moribund; 5, dead (Jones et al., 2008).

Histological evaluation of spinal cord and brain

On day 21 after the initial immunization, mice were transcardially perfused with 4% paraformaldehyde (PFA) in urethane anesthesia. The brain and spinal cord were removed and post-fixed in the same fixative. Paraffin-embedded sections at 5 μm were made after embedding in paraffin and then stained with Luxol Fast Blue (LFB) to detect demyelination. Frozen sections at 30 μm were stained with Cresyl violet to determine inflammatory cell infiltration.

Measurement of demyelination

To quantify demyelination, Luxol Fast Blue (LFB) staining was performed with 5 μm thick thoracic spinal cord sections from normal or EAE mice at 21 days after the initial immunization. From the spinal cord section images, the demyelinated area was measured using Photoshop (Adobe Photoshop CS4). The region of spinal cord in the whole image was selected, and then demyelinated area was expressed as % area.

Immunohistochemical examination of the spinal cord

The spinal cord, previously perfused with 4% paraformaldehyde, was post-fixed in the same fixative overnight and cryoprotected in PBS containing 30% sucrose at 4 °C for 2 days. Frozen 30 μm sections of the spinal cord were immunohistochemically stained with specific antibodies against cell surface molecules for cluster differentiation (CD) according to conventional methods. Monoclonal antibodies against CD4 (BD Bioscience, CA), CD8 (BD Bioscience, CA), F4/80 (eBioscience, CA) produced in a rat or a polyclonal antibody against CD20 produced in goat (SantaCruz Biotechnology, CA) were used as the primary antibodies. An antibody against rat IgG or goat IgG (Vector, Burlingame, CA) was employed as the secondary antibody. The immune reaction was visualized with 3,3′-diaminobenzidine (DAB) after incubation in ABC reagent (Vector, Burlingame, CA). To test whether synaptic vesicular zinc is involved in the formation of aberrant zinc patches, an affinity-purified rabbit antibody specific for ZnT3 (1:100, provided by Dr. R. Palmer) was used for immunohistochemical localization. For counterstaining of microglia and neurons, antibodies against F4/80...
(1:500) and MAP2 (1:200) were used, respectively. The immunolabeling procedures were performed as per routine immunostaining protocols. For visualizing primary antibody binding, the following secondary antibodies were used: Alexa594 (ZnT3), Alexa488 (MAP2 and F4/80). Stained sections were rinsed in PBS. Finally they were mounted on 0.5% gelatin-coated slides, coverslipped and photographed with an Olympus fluorescence microscope. To assess nonspecific effects a few sections in every experiment were incubated in a buffer without primary antibodies. This procedure always resulted in a complete lack of immunoreactivity.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

The spinal cord was perfused with cold PBS and extraction of total RNA was performed with the Trizol reagent (Invitrogen, CA). RT-PCR was performed according to the conventional method. The oligonucleotide primers were as follows: CD4 forward 5′-TGTGCCAGCCATGTGAA-3′; reverse 5′-GCTGGCCTGGAGGCACTG-3′; β-actin forward 5′-TGAAT CCTTGGCACCATTGAAAAC-3′, reverse 5′-TAAACGCACGTCGTACG-3′. PCR products were separated on an agarose gel and then bands were quantified using Image J (NCBI, MD) after staining with ethidium bromide.

Zinc staining

The N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ) histochcmical method was used as previously described (Frederickson et al., 1987; Suh et al., 1999). Briefly, mice were sacrificed at the designated time points by decapitation under 5% isoflurane anesthesia and the spinal cords were removed and frozen in powdered dry ice. The frozen, unfixed spinal cords were coronally sectioned at 25 μm thickness in a -20 °C cryostat, then thawed onto gelatin-coated slides and air-dried.

Fig. 1. CQ ameliorates the clinical signs and disease progression of myelin oligodendrocyte glycoprotein (MOG) induced EAE. Clinical disease scores were recorded daily until 21 days after induction. The clinical signs of EAE first appeared on day 13 and reached peak levels on day 19 from the 1st MOG (M) immunization. CQ (M+CQ) or its vehicle (M+DMSO) was orally administered during the entire period. Control groups also received oral CQ (N+CQ) or vehicle only (N+DMSO) without MOG immunization. (A) CQ profoundly reduced the EAE clinical score induced by MOG. Data are mean ± s.e.m. (n=14–21) *p<0.05 compared with CQ treated group. (B) CQ reduced the incidence rate of EAE induced by MOG. (C,D) CQ reduces MOG-induced demyelination in spinal cord of EAE mice. Mice were sacrificed 21 days after MOG injection and sections of spinal cord were evaluated for damaged axons using Luxol Fast Blue (LFB). On day 21 after the initial immunization, demyelination in the spinal cord was visualized with LFB staining. White matter lesioning is reflected by reduced LFB staining in the spinal cord. (C) LFB staining of the spinal cord shows extensive demyelination in the spinal cord of EAE mice. EAE-associated demyelination is almost completely absent in EAE mice treated with CQ. The square area in the low magnification of left panel was enlarged four hundred times and illustrated on right panel. Scale bar represents 100 μm. (D) A graph represents percent of demyelination of white matter of thoracic spinal cord with or without CQ treatment in EAE mice. Data are mean ± s.e.m. (n=3) *p<0.05 compared with CQ treated group.
The sections were immersed in a solution of 4.5 μM TSQ (Molecular Probes, Eugene, OR) in 140 mM sodium barbital and 140 mM sodium acetate buffer (pH 10.5–11) for 60 s, and then rinsed for 60 s in 0.9% saline. TSQ binding was imaged with a fluorescence microscope (Olympus upright microscope, epi-illuminated with 360 nm UV light) and photographed through a 500 nm long-pass filter using a INFINITY 3-1 camera (Lumenera Inc., ON, Canada) with INFINITY analyze software (Lumenera Inc., ON, Canada).

Detection of blood–brain barrier disruption by IgG extravasation

To detect blood–brain barrier disruption, histological analysis of IgG extravasation was performed with thoracic spinal cord of normal or EAE mice at 21 days after first MOG injection. Free-floating 30 μm coronal sections from the spinal cord were immunostained with biotinylated horse anti-mouse lgG (Vector, Burlingame, CA). From the spinal cord section images, the IgG stained area was also measured by Image J. Measurement of the IgG stained area was quantified using the method modified from Tang et al. (2010). Briefly, to quantify the area of IgG leakage, the image was loaded into Image J and converted into an 8-bit image. Then, the image was thresholded using the menu option. The type was set to Black & White and the bottom slider moved to a value of 106. The resulting thresholded image is binary and will only show the region of IgG leakage. To measure this area, the menu option Analyze → Measure was selected. The selected part of spinal cord in the whole image was sorted, and then the area of IgG leakage was expressed as % area.

Detection of MMP-9 activation by in situ zymography

To detect matrix metalloproteinases-9 (MMP-9) activation, we used in situ zymography to localize net gelatinolytic activity in spinal cord sections (Rivera et al., 2002). Frozen, non-fixed 25 μm spinal cord sections were thawed and incubated for 3 h at 37 °C in a humid, dark chamber in reaction buffer containing 25 μg/ml of FITC-labeled DQ-gelatin (EnzChek gelatinase/collagenase assay kit, Molecular Probes, Eugene, OR). The sections were rinsed in PBS for 10 min and fixed in 4% paraformaldehyde for 20 min then mounted in fluorescent mounting medium (VECTOR, Burlingame, CA). Cryostat sectioned spinal cord were photographed with a fluorescent microscope. From the spinal cord section images, MMP-9 stained area was measured by Image J as described above.

Detection of autophagy induction

To identify autophagy induction in EAE spinal cord, double immunofluorescence staining was performed. Thoracic spinal cord sections were incubated in mixture of rabbit anti-LC3 (diluted 1:200, Novus, CT)/mouse anti-MAP2 (diluted 1:200, Millipore, Billerica, MA) in

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Fig. 2. CQ treatment attenuates EAE-induced mononuclear cell infiltration into the white matter of spinal cord and cerebellum in mice. Three weeks after the initial immunization of MOG, infiltration of mononuclear cells around small vessels in the spinal cord and cerebellum was detected with Cresyl violet staining. EAE mice revealed intensive infiltration of mononuclear cells around the white matter of spinal cord (A) and cerebellum (C). However, CQ treatment reduces mononuclear cell infiltration into the white matter of the spinal cord. Scale bar represents 100 μm. Graph represent number of infiltrated cells in the white matter of thoracic spinal cord (B) and cerebellum (D) with or without CQ treatment in EAE mice. Data are mean±s.e.m. (n=3) * p<0.05 compared with CQ treated group.
Western blot analysis

Spinal cord samples were homogenized in RIPA buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS. The homogenate was then centrifuged at 16,000×g for 20 min at 4 °C and the supernatant was retained and preserved at −70 °C for later use. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Sigma, St Louis, MO). Ten micrograms of protein from each sample was separated by electrophoresis on 15% SDS-PAGE gel. The separated proteins were transferred to PVDF membranes (Millipore, Billerica, MA) which were blocked with Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% skim milk for 1 h at RT. After blocking, the membranes were incubated with primary antibodies against LC3 (Novus Biologicals, Littleton, CO, 1:10,000), α-tubulin (abcam, UK, 1:5000) in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% skim milk for overnight at 4 °C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at RT. Immunoreactivity was detected with enhanced chemoluminescent autoradiography (ECL kit, GE Healthcare, UK), according to the manufacturer’s instructions. Immunoreactivity was analyzed by densitometry using Image J (NCBI, MD) and was normalized to a loading control, α-tubulin.

Statistical analysis

EAE clinical scores were reported as mean ± s.e.m. Repeated measure analysis of variance (ANOVA) was used for statistical analysis of these data. The fluorescence intensity and T-cell proliferation data were expressed as the mean ± s.e.m. and analyzed for statistical significance using one-way ANOVA, followed by a Bonferroni post hoc test. p<0.05 was considered significant.

Results

CQ ameliorates clinical signs of MOG-induced EAE and disease progression

Mice immunized with MOG(35–55) developed severe EAE symptoms with complete hind limb paralysis (EAE incidence rate 21/21). As shown in Fig. 1, consecutive administration of CQ from beginning of MOG injection resulted in amelioration of the clinical score (Fig. 1A) and incidence rate (Fig. 1B) of EAE. CQ treatment decreased the motor deficit score at 19 days from 2.38 ± 0.55 (of 5 maximum) to 0.79 ± 0.67, a 67% reduction (Fig. 1A).

CQ reduces MOG-induced white matter damage in spinal cord of EAE mice

LFB staining of the spinal cord revealed extensive demyelination in the MOG-immunized EAE mice (Fig. 1C). In contrast, CQ-treated EAE mice showed much less demyelination. Compared with the vehicle-treated EAE group, the CQ-treated EAE group had an 83% reduction in demyelination in the examined spinal cord sections (Fig. 1D).
Fig. 4. CQ reduces formation of aberrant zinc patches, blood-brain barrier disruption and MMP-9 activation in the white matter of EAE spinal cord. (A) Fluorescence images represent chelatable zinc staining by TSQ in mouse spinal cord. TSQ zinc staining was performed at 21 days after the MOG injection. “Sham” operated (with or without CQ injection) spinal cord section shows bright fluorescence in the gray matter but almost no fluorescence in the white matter. However, EAE mice spinal cord shows aberrant TSQ staining in the white matter. This abnormal TSQ staining represents “patch-like” fluorescence in white matter indicative of abnormal synaptic zinc accumulation in this area. Mice receiving oral CQ after MOG injection showed substantially reduced TSQ staining in the white matter. Scale bar represents 100 μm. (B) To test whether synaptic vesicular zinc is involved in the formation of aberrant zinc patches, synaptic zinc terminal (ZnT3), microglia (F4/80) and neurons (MAP2) were immunohistochemically stained. Images show that vesicular zinc terminals and neuron terminals were co-localized in the patches. However, microglia and ZnT3 were not co-localized in the patches. Scale bar represents 50 μm. (C,D) Images represent immunohistochemical characterization of IgG extravasation in the thoracic spinal cord of EAE mice. Sham-operated spinal cord section showed only weak IgG immunoreactivity (IR) primarily confined to the BBB-deficient area. Some perivascular extravasation of IgG (brown color) was detected in the thoracic spinal cord of normal mice. Prominent extravasation of IgG was seen throughout the parenchyma of the spinal cord 21 days after MOG injections (Vehicle, Veh). However, the extensive diffusion of IgG IR was reduced by CQ injection (CQ) in EAE mice. Scale bar represents 100 μm. (D) A graph represents percent area of IgG IR of white matter of thoracic spinal cord with or without CQ treatment in EAE mice. Data are mean±s.e.m. (n=3) *p<0.05 compared with CQ treated group. (E,F) Net in situ zymography was increased after MOG injection. Fluorescence photographs of spinal cord sections showing in situ gelatinolytic activity in the white matter of sham operated or of EAE mice. A weak fluorescence signal was detected in “Sham” operated animals. However, the fluorescence intensity increased in the white matter of the spinal cord in EAE mice. This increased fluorescence intensity was reduced in CQ treated mice. Scale bar represents 100 μm. (F) A graph represents percent area of in situ gelatinolytic activity of white matter of thoracic spinal cord with or without CQ treatment in EAE mice. Data are mean±s.e.m. (n=3 each) *p<0.05 compared with CQ treated group.
from EAE mice were rich in CD4+ T and CD8+ T cell infiltrates, while treatment with CQ significantly decreased CD4+ T and CD8+ T cell infiltrates into the white matter (Fig. 3A). F4/80 expression was apparently increased on day 21 post-MOG immunization. In contrast, a significantly reduced number of F4/80 stained cells were present after CQ treatment (Fig. 3A).

CQ treatment suppresses expression of CD4, CD8, CD11b and CD20 mRNA in the spinal cord of EAE mice

RT-PCR studies on expression of mRNA of cell surface molecules also exhibited that mRNA expression of CD4, CD8, CD11b and CD20 was coincidentally up-regulated in the spinal cords of EAE mice (Figs. 3B, C). Remarkably, CQ also inhibited the EAE-associated increase in mRNA expression of these species.

CQ reduces formation of aberrant zinc patches in the white matter of EAE spinal cord

TSQ staining of spinal cords harvested at 21 days after the MOG injection showed intense “patch-like” fluorescence in white matter of spinal cords (Fig. 4A), indicative of abnormal zinc accumulation in this area. Mice receiving oral CQ after MOG injection showed substantially reduced TSQ staining in the white matter. To test whether synaptic vesicular zinc is involved in the formation of aberrant zinc patches, synaptic zinc-containing terminals (ZnT3), microglia (F4/80) and neurons (MAP2) were immunohistochemically stained. In the present study we found that vesicular zinc-containing terminals and neuronal terminals were co-localized in the patches. However, microglia and ZnT3 were not co-localized in the patches (Fig. 4B).

CQ decreases MOG-induced blood–brain barrier disruption

To evaluate the putative damage of the BBB, we looked for a leakage of serum IgGs using immunohistochemistry. Compared with controls, there was a significant increase in IgG extravasation observed in the thoracic spinal cord at 21 days after MOG injection. Compared to the MOG-only injection group, CQ-injected subjects showed significantly reduced IgG extravasation (Figs. 4C, D). We also performed a conventional method to evaluate BBB disruption with Evans Blue at 21 days after first MOG injection. We found that Evans Blue leakage also showed similar results as seen using IgG staining (Supplemental Figure 1).

CQ decreases MOG-induced MMP-9 activation

In order to determine whether net MMP9 gelatinolytic activity was increased in the EAE spinal cord, we used in situ zymography in ex vivo spinal cord slices. Twenty-one days after MOG injection, strong increases in fluorescent signal were detected in the white matter of spinal cord (MOG+Vehicle). In contrast, a significant decrease in

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**Fig. 5.** MOG injection promotes autophagy induction, which is enhanced by CQ. To assess the potential role of autophagy in multiple sclerosis (EAE), thoracic spinal cords were investigated. To test whether CQ treatment influences autophagy induction in EAE mice, we used immunohistochemistry (A,B) and western blot (C–E). (A) Green fluorescent images show microtubule-associated protein 2 (MAP2) and red fluorescent images show autophagy microtubule-associated protein light chain 3 (LC3) staining. Compared to “Sham” operated control, immunohistochemically stained spinal cord shows increased LC3 intensity in motor neuron of EAE mice. LC3-positive signals were co-localized with neuronal cell body (MAP2). CQ treatment further increased LC3 fluorescence intensity. Scale bar represents 50 μm. (C–E) Unfixed spinal cords were used for western blot to analyze LC3 at 21 days after MOG injection. Data represent elevated levels of LC3 in EAE mice (MOG+Vehicle), which was further increased by CQ treatment (MOG+CQ). (D) Graph represents quantitated LC3-II expression level (fold of increase) with or without CQ treatment in EAE mice. Data are mean±s.e.m. (n=3). *p<0.05 compared with each group. (E) Graph represents quantitated Beclin-1 expression level (fold of increase) with or without CQ treatment in EAE mice. Data are mean±s.e.m. (n=3). *p<0.05 compared with each group.
fluorescence intensity was observed in CQ-treated spinal cord (MOG + CQ) (Figs. 4E, F).

**MOG injection promotes autophagy induction, which is enhanced by CQ**

To test whether CQ treatment influences autophagy induction in EAE mice, we used immunohistochemistry (Figs. 5A, B) and western blot analysis (Figs. 5C, D, E). Compared to “Sham” operated control, immunohistochemically stained spinal cord shows increased autophagy microtubule-associated protein light chain 3 (LC3) intensity. CQ treatment further increased LC3 fluorescence intensity in EAE mice. Next we used western blot analysis to investigate biochemical process for autophagy induction in EAE mice. The expression of LC3 II and Beclin-1 protein was significantly increased at 21 days after MOG injection (Figs. 5C, D, E, p < 0.05). Administration of CQ increased the relative protein abundance of LC3 II comparing with EAE mice.

**Post-treatment of CQ also ameliorates clinical signs and histological progress of EAE**

To test whether post-treatment of CQ also effective on EAE disease progression, CQ was delivered three days after the typical onset of clinical symptom (15 days after MOG injection). As shown in above experiments, post-treatment of CQ also reduced clinical score and histological findings. As shown in Fig. 1, post-treatment with CQ also produced improvement of the clinical score (Fig. 6A) and reduced demyelination (Fig. 6B) of EAE animals, compared to the vehicle treated group. CQ treatment decreased the motor deficit score at 19 days from 2.1 ± 0.66 (of 5 maximum) to 0.9 ± 0.27, a 63% reduction (Fig. 6A). And CQ treatment also decreased the demyelination (Fig. 6B). Furthermore, this preservation of the clinical score was evident until at least 30 days after first MOG immunization.

**Discussion**

The present study shows that oral administration of CQ, a cell permeable metal chelator, ameliorated clinical signs of MOG-induced EAE. The attenuation of EAE progression by CQ was accompanied by reduced white matter damage, immune cell infiltration, BBB disruption, and enhanced induction of autophagy.

Zinc is involved in immune processes and several neurodegenerative diseases including Alzheimer’s disease (AD), Huntington’s disease and Parkinson’s disease (Frederickson et al., 2005). Brain zinc dyshomeostasis is a prominent feature of advanced AD, where zinc is biochemically linked to brain amyloid beta-peptide accumulation and dementia severity (Bush et al., 1994; Cherny et al., 2001; Suh et al., 2000b). Like zinc, copper concentration in the brain is also high and may be released into the synaptic space by neuronal activity (Hartter and Barnea, 1988; Kardos et al., 1989). Although altered serum or plasma concentrations of copper and zinc have been reported in multiple sclerosis (Kapaki et al., 1989; Melo et al., 2003),

![Fig. 6. Post-treatment of CQ also ameliorates the clinical signs and disease progression of EAE. Clinical disease scores were recorded daily until 30 days after induction. Clinical signs of EAE first appeared on day 13 and reached peak levels on day 19 from the 1st MOG (M) immunization. CQ (M + CQ) or its vehicle (M + DMSO) was administered orally from day 15 from the 1st MOG (M) immunization. Control groups also received CQ (N + CQ) or vehicle only (N + DMSO) without MOG immunization. (A) Post-treatment with CQ profoundly reduced the EAE clinical score induced by MOG. Data are mean ± s.e.m. (n = 5–6) * p < 0.05 compared with CQ treated group. (B) Post-treatment with CQ reduces MOG-induced demyelination in the spinal cord of EAE mice. Mice were sacrificed 30 days after MOG injection and sections of spinal cord were evaluated for damaged axons using Luxol Fast Blue (LFB). On day 30 after the initial immunization, demyelination in the spinal cord was visualized with LFB staining. White matter lesioning is reflected by reduced LFB staining in the spinal cord. LFB staining of the spinal cord shows extensive demyelination in the MOG-immunized EAE mice. EAE-associated demyelination is significantly less in EAE mice treated with CQ. Scale bar represents 100 μm.](image-url)
the number of studies performed to investigate the modulating effects of these transition metals on MS has been sparse.

CQ is a metal chelator that acts on transition elements, including zinc and copper. Recent animal studies have shown that CQ can reverse the progression of Alzheimer's, Parkinson's, and Huntington's diseases (Cherny et al., 2001; Kaur et al., 2003; Nguyen et al., 2005). Evidence from clinical trials suggests that CQ may halt cognitive decline in Alzheimer's disease, possibly owing to its ability to act as a chelator for zinc and copper ions (Ritchie et al., 2003). CQ treatment of transgenic Huntington's mice decreased Huntington's aggregate accumulation, decreased striatal atrophy, improved rotaorad performance, and extended lifespan (Nguyen et al., 2005). Recent studies have demonstrated that CQ acts as a zinc ionophore under physiological conditions. Park et al. presented a study that CQ induces autophagy in a zinc-dependent manner and contributes to clearance of aggregated proteins in astrocytes and neurons. Hence, in addition to its metal-chelating effects in and around amyloid beta (Aβ) plaques, CQ may contribute to the reduction of Aβ loads by activating autophagy via increasing or normalizing intracellular zinc levels in the brain (Park et al., 2011).

Zinc has been reported to affect the function of various immune cells, including T cells, B cells, natural killer cells, microglia and macrophages in rodents and humans (Prasad, 2000; Rink and Gabriel, 2000). Our previous study suggested that zinc chelation reduced ischemia-induced microglia activation, which may prevent later neuron death (Kauppinen et al., 2008). Recent studies have suggested that copper is also associated with T cell mediated autoimmune neuroinflammation. The copper chelator, cuprizone, attenuated clinical severity and progression of EAE, which suggested that copper may also be involved in the mechanism of T cell mediated disease progression (Emerson et al., 2001; Mana et al., 2009). The present study shows that CQ administration diminished the activation of microglia in the spinal cord of EAE mice. The near-complete suppression of microglial activation in the spinal cord treated with CQ in the present study suggests that endogenous zinc/copper release is an early signal triggering microglial activation in EAE. Thus, by silencing microglial cells, CQ may be able to prevent further inflammatory processes within the CNS parenchyma, that is, myelin antigen exposure, T cell infiltration, cytokine release and recruitment of other immune cells. The present study shows that CQ injection reduced the infiltration of CD4+, CD8+ T cells and CD20 cells in the spinal cord of MOG-induced EAE mice. Thus, by silencing microglial cells, CQ may be able to prevent T cell infiltration within the CNS parenchyma during EAE. Since CQ was injected into the oral, CQ could affect not only central nervous system but also act systemically. In the present study, we couldn't delineate this effect. However, we believe that systemic injection of CQ may also decrease pre-T-cell survival and differentiation into Th1 cells (Csermely and Somogyi, 1989; Rigas et al., 1979).

Normally, the central nervous system is not accessible to circulating white blood cells due to the blood–brain barrier (BBB). However, during an MS attack, the BBB becomes compromised in the brain or spinal cord, allowing T lymphocytes to cross and attack antigens present on myelin (Zlokovic et al., 1989). Matrix metalloproteinase-9 (MMP-9) plays a role in MS, alluding to that possibility that its activity may mediate T cell migration across the subendothelial basement membrane. Furthermore, MMP-9 may contribute to myelin breakdown due to its proteolytic activity against myelin basic protein (Leppert et al., 1998). A previous study showed that MMP-9 levels are strongly elevated during demyelination and MMP-9 knockout mice are resistant to EAE in the early stages of disease (Dubois et al., 1999). It is possible that the damaged BBB permits the passage of the trace elements Zn and Cu, which may aggravate several downstream events including immune reaction and zinc-dependent metalloproteinases (MMPs) activation or vice versa. MMPs are a family of zinc- or copper-dependent neutral endopeptidases, collectively capable of degrading essentially all matrix components. Our present study shows that CQ stabilizes BBB breakdown and IgG leakage through MMP-9 inhibition in the spinal cord of EAE, which suggests that zinc and copper may be involved in MMP-9-induced BBB breakdown in MS.

Factors involved in the process of autophagy have been implicated in a number of neurodegenerative diseases (Alirezaei et al., 2008; Kundu and Thompson, 2008). Autophagy is a cellular degradation process for the removal of damaged intracytosolic organelles through fusion with lysosomes (Klionsky and Emr, 2000). Recently, Park et al. found that CQ induces autophagy in a zinc-dependent manner and contributes to clearance of aggregated proteins in astrocytes and neurons (Park et al., 2011). Thus, we examined whether CQ could induce autophagy in the spinal cord of EAE mice. We found the levels of autophagy were increased in the spinal cord motor neurons after MOG injection, as seen in a previous study (Alirezaei et al., 2009) and that CQ further increased the level of autophagy induction. These results suggest that autophagy may contribute to removal of degraded or damaged intracellular organelles in spinal cord neurons of EAE mice. Hence, in addition to metal chelating effects, CQ may...
contribute to the reduction of damaged proteins by activating autophagy via normalized intracellular metal concentrations in spinal cord neurons (Parkers, 2011).

In summary, CQ ameliorates EAE-induced clinical sign of MS in mice. This amelioration was accompanied by inhibition of the EAE-associated demyelination and infiltration of encephalitogenic immune cells including T cells, B cells and microglia in the CNS. CQ treatment also showed reduction of BBB breakdown by inhibition of MMP-9, which is likely a key step by which CQ prevents immune cell infiltration. CQ treatment may also derive its therapeutic effect from enhancing clearance of damaged cellular components by autophagy as illustrated in Fig. 7. Furthermore, the present study found that post-treatment with CQ also reduced the clinical score of EAE and spinal cord demyelination. Therefore, taken together, our results suggest that transition metal ions, including zinc and copper, are involved in several steps of MS pathogenesis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2013.01.012.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (2012R1A2A2A01040132). We would like express our special thanks to Mr. Jae Nam Seo for his excellent technical assistance. Ms. Tae Yul Kim helped us with excellent illustrations.

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