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Hydroxychloroquine reduces microglial activity and attenuates experimental autoimmune encephalomyelitis

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ABSTRACT

Background: Microglial activation is thought to be a key pathophysiological mechanism underlying disease activity in all forms of MS. Hydroxychloroquine (HCQ) is an antimalarial drug with immunomodulatory properties that is widely used in the treatment of rheumatological diseases. In this series of experiments, we explore the effect of HCQ on human microglial activation in vitro and on the development of experimental autoimmune encephalitis (EAE) in vivo.
Methods: We activated human microglia with lipopolysaccharide (LPS), and measured concentrations of several pro- and anti-inflammatory cytokines in untreated and HCQ pretreated cultures. We investigated the effect of HCQ pretreatment at two doses on the development of EAE and spinal cord histology.
Results: HCQ pretreatment reduced the production of pro-inflammatory (TNF-alpha, IL-6, and IL-12) and anti-inflammatory (IL-10 and IL-1 receptor antagonist) cytokines in LPS-stimulated human microglia. HCQ pretreatment delayed the onset of EAE, and reduced the number of Iba-1 positive microglia/macrophages and signs of demyelination in the spinal cords of HCQ treated animals.
Conclusion: HCQ treatment reduces the activation of human microglia in vitro, delays the onset of EAE, and decreases the representation of activated macrophages/microglia and demyelination in the spinal cord of treated mice. HCQ is a plausible candidate for further clinical studies in MS.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the brain and spinal cord that leads to disability and functional loss due to demyelination and neuronal injury [1]. Although the cause of MS is unknown, pathological research has shown that inflammation is the hallmark of all forms of MS, and that activated microglia and phagocytic macrophages are important participants in this pathology. Microglial activation is present in all types of MS plaques [2] and also in the extralesional normal appearing white matter (NAWM) [3–5]. While there are beneficial activities of microglia [6], abnormally activated microglia produce a variety of molecules that can destroy neurons and oligodendrocytes, including free radicals, proteases and glutamate [7]. Indeed, the chronic activation of microglia and the persistence of their toxic products are thought to drive the progressive destruction of axons in MS and its animal models [4,8].

Current treatments for MS only impact the most common subtype of MS, relapsing–remitting MS (RRMS), while no treatments so far have shown a convincing effect on primary and secondary progressive MS. One strategy in the search for treatments for all forms of MS, but in particular for the currently untreatable progressive forms of MS, is the application of generic drugs to MS. The underlying thought of this approach is to screen an available generic drug for its effect on a pathophysiological mechanism thought to be important in MS, and to test this generic drug in a clinical trial. For example, a recent effort to screen generic drugs for their influence on oligodendrocyte differentiation and remyelination led to the identification of the generic antihistamine clemastine as a candidate drug to promote remyelination [9], and to a phase II trial of this agent in RRMS (Clinicaltrials.gov reference NCT02040298). Current treatments for MS do not directly target microglia. However, given the prominence of microglial activation in the pathology of RRMS as well as progressive MS, drugs that target microglial activation could have an important impact on the pathophysiology of all forms of MS.

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Hydroxychloroquine (HCQ) is an antimalarial drug with immunomodulatory effects that is widely used in combination with other disease suppressing medications in the treatment of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [10,11]. Long term use of HCQ as maintenance or adjunct treatment in SLE has been shown to reduce occurrence of disease exacerbations, and it may delay development of neuropsychiatric features [12]. While its precise mode of action in these diseases is uncertain it is notable that HCQ can inactivate macrophage phospholipase A2 and reduce production of pro-inflammatory cytokines by macrophages and lymphocytes [13,14]. In addition to immunological actions, HCQ has antithrombotic, lipid-lowering and other metabolic actions [10,11]. Interestingly, with prolonged treatment HCQ tends to accumulate in tissues including the brain [15], and it might therefore be effective in the treatment of MS, where the blood–brain barrier often forms an obstacle to achieving sufficient drug levels. We conducted a series of experiments to test HCQ as an inhibitor of microglial activation in vitro, and to investigate its effects on experimental autoimmune encephalomyelitis (EAE), an animal model of MS.

2. Methods

2.1. Preparation and treatment of human microglia

Human microglia of over 95% purity was isolated from the brains of adult humans undergoing resection to treat intractable epilepsy, as previously described [16]. The use of these specimens was approved by the University of Calgary Research Ethics Board. Cells were plated in 96-well flat-bottomed plates (BD Pharmingen, San Jose, USA) at a density of 10,000 cells per well. The feeding medium used was minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 0.1% dextrose, nonessential amino acids, 10 μM glutamine and 1 mM sodium pyruvate (called complete MEM). Although microglia are known to be cells that react to perturbations in vivo, the human microglia in culture appear to be at a very low basal level of activation, as evident by the minimal levels of secreted cytokine molecules in control, unstimulated condition (see vehicle-treatment condition of Fig. 2).

Where indicated, adherent cells were treated with 100 ng/ml of the Toll-like receptor-4 agonist lipopolysaccharide (LPS), and with varying concentrations of HCQ. All treatments with HCQ were done 2 h prior to the addition of LPS, except where stated otherwise; this pretreatment period was necessary because LPS is a potent activator that triggers signaling cascades immediately after engagement of Toll-like receptors on cells. All chemicals were obtained from Sigma-Aldrich (St. Louis, USA). Cell-conditioned media were collected after 24 h and used for cytokine analyses.

2.2. Cytokine analyses

Tumor necrosis factor (TNF)-alpha concentrations in the microglia culture medium were measured with a single cytokine TNF-alpha ELISA (ELISA Kit KHC3011, Invitrogen, Carlsbad, USA). The concentration of 25 cytokines and chemokines was also measured simultaneously in the microglia medium with a multiplex human cytokine panel (Kit LHC0009, Invitrogen, Carlsbad, USA). The cytokines measured with the multiplex panel were granulocyte-macrophage colony stimulating factor, interferon (IFN)-alpha, IFN-gamma, interleukin (IL)-1 receptor antagonist (ra), IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15 IL-17 and TNF-alpha. Chemokines measured were IFN-gamma-inducible protein 10 (IP10), monocyte chemoattractant protein 1 (MCP-1), monokine induced by IFN-gamma (MIG), macrophage inflammatory protein 1alpha (MIP-1alpha), MIP-1beta, RANTES and eotaxin.

2.3. EAE disease induction and analyses

EAE was induced in female C57BL/6 mice (Charles River, Montreal, Canada), ages 8–10 weeks, by subcutaneous injection of 50 μg of $MOG_{35–55}$ in Freund's Complete Adjuvant (Thermo Fisher Scientific, Rockford, USA) supplemented with 4 mg/ml of Mycobacterium tuberculosis on day 0. Intraperitoneal pertussis toxin (0.1 μg/200 μl; List Biological Laboratories, Hornby, Canada) was administered at days 0 and 2. To increase the sensitivity of measurement, animals were assessed daily using a 15 point disease score scale [17,18] instead of the more commonly used 5 point scale. The 15 point scale score (0 to 15) is the sum of the disease state for the tail (scored from 0 to 2) and each limb (scored from 0 to 3); death is scored as 15. All animals were handled according to the policies outlined by the Canadian Council for Animal Care and the University of Calgary. HCQ was dissolved in phosphate buffered saline and injected intraperitoneally in a volume of 200 μl on the days and in the dosages noted.

2.4. Histological analysis

Animals were killed with an overdose of ketamine/xylazine (200 and 10 mg/kg, respectively). Spinal cords were removed, fixed in 10% buffered formalin and the lumbar region then embedded in paraffin. Longitudinal sections of 6 μm thickness were cut from the ventral to dorsal aspects of the lumbar spinal cord. Sections were stained with

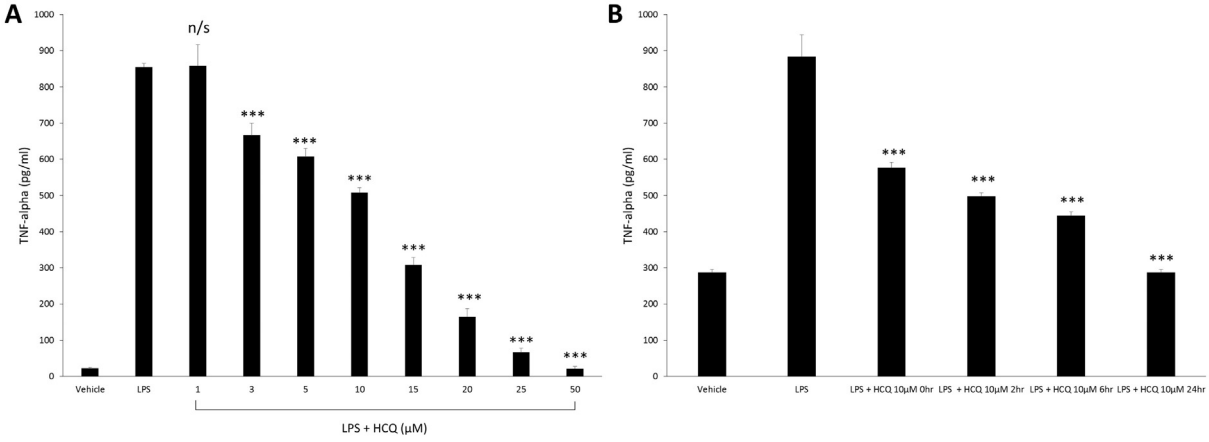

Fig. 1. A) HCQ reduces the production of TNF-alpha in LPS-activated microglia in a concentration-dependent manner. B) The inhibition of activated microglia is stronger the longer the pretreatment interval with HCQ; the times indicated are hours prior to the addition of LPS. All samples (culture media) were collected for ELISA determination 24 h after the addition of LPS. Values are mean ± SEM of 4 wells of microglia per condition.

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