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Protective effects of monomethyl fumarate at the inflamed blood–brain barrier

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ABSTRACT

Background: Reactive oxygen species play a key role in the pathogenesis of multiple sclerosis as they induce blood–brain barrier disruption and enhance transendothelial leukocyte migration. Thus, therapeutic compounds with antioxidant and anti-inflammatory potential could have clinical value in multiple sclerosis. The aim of the current study was to elucidate the therapeutic effects of monomethyl fumarate on inflammatory-mediated changes in blood–brain barrier function and gain insight into the underlying mechanism.

Methods: The effects of monomethyl fumarate on monocyte transendothelial migration across and adhesion to inflamed human brain endothelial cells (hCMEC/D3) were quantified using standardized in vitro migration and adhesion assays. Flow cytometry analysis and qPCR were used to measure the concomitant effects of monomethyl fumarate treatment on protein expression of cell adhesion molecules. Furthermore, the effects of monomethyl fumarate on the expression and nuclear localization of proteins involved in the activation of antioxidant and inflammatory pathways in human brain endothelial cells were elucidated using nuclear fractionation and Western blotting. Statistical analysis was performed using one-way ANOVA followed by the Bonferroni post-hoc test.

Results: Our results show that monomethyl fumarate induced nuclear translocation of nuclear factor (erythroid-derived 2)-like 2 and concomitant production of the antioxidant enzymes heme oxygenase-1 and NADPH:quinone oxidoreductase-1 in brain endothelial cells. Importantly, monomethyl fumarate treatment markedly decreased monocyte transendothelial migration across and adhesion to inflamed human brain endothelial cells. Treatment of brain endothelial cells with monomethyl fumarate resulted in a striking reduction of vascular cell adhesion molecule expression. Surprisingly, monomethyl fumarate did not affect nuclear translocation of nuclear factor-κB suggesting that monomethyl fumarate potentially affects activity of nuclear factor-κB downstream of nuclear translocation.

Conclusions: Taken together, we show that monomethyl fumarate, the primary metabolite of dimethyl fumarate, which is currently used in the clinics for the treatment of relapsing–remitting multiple sclerosis, demonstrates beneficial therapeutic effects at the inflamed blood–brain barrier.

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) and is histopathologically characterized by focal demyelinated lesions, immune cell infiltration, oligodendrocyte death, neuro-axonal damage and astrogliosis (Brück, 2005; Frohman et al., 2006). Infiltration of peripheral immune cells through a disrupted blood–brain barrier (BBB) into the CNS is a pivotal and early event in the disease process. Under normal conditions, the BBB forms an anatomical and physiological barrier between the CNS and the systemic circulation. Specialized brain endothelial cells (BECs) lining the vessel wall are connected by tight-junctions, thereby minimizing the entry of immune cells from the blood into the CNS. In MS, however, activated monocytes produce a range of inflammatory mediators, including nitric oxide, reactive oxygen species (ROS) and tumor necrosis factor-α (TNF) that affect the immune quiescence of the BBB, thereby enhancing adhesion and migration of leukocytes. In particular, TNF was shown to promote transendothelial leukocyte migration by upregulation of vascular adhesion molecules (Barten and Ruddle, 1994; Male et al., 1994; Wong et al., 1999). Importantly, TNF induces ROS production by BECs (Gu et al., 2002; Li et al., 2005; Basuroy et al., 2006; Tang et al., 2011; Rochfort et al., 2014), which further impairs BBB integrity. Our previous works also demonstrated

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that ROS are produced upon interaction of monocytes with BECs (van der Goes et al., 2001; Schreibelt et al., 2006). The importance of ROS in BBB dysfunction has been further demonstrated by studies using antioxidant therapies that resulted in decreased monocyte migration across BECs and an ameliorated disease course in the experimental autoimmune encephalomyelitis (EAE) animal model for MS (van der Goes et al., 2001; Schreibelt et al., 2006; Hendriks et al., 2004). Taken together, these findings show that antioxidant compounds may be an important therapeutic strategy for BBB dysfunction and monocyte infiltration in MS.

In recent years, significant advances in the development of disease modifying treatments for relapsing–remitting MS have been made, and include a therapeutic compound that can induce the endogenous antioxidant response, namely Tecfidera™. An oral formulation, Tecfidera™ has demonstrated efficacy in two phase 3 trials and contains dimethylfumarate (DMF), which is after absorption rapidly converted into the bioactive compound monomethyl fumarate (MMF). Fumarates are able to activate the nuclear factor erythroid-2 related factor-2 (Nrf2) pathway (Lin et al., 2011; Scannevin et al., 2012). This endogenous cellular stress response involves the transcription of multiple (antioxidant) proteins, including heme-oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO-1) and enzymes involved in glutathione synthesis, that protect cells from ROS-induced damage and cell death. Deletion of Nrf2 in mice abrogated the beneficial effects of DMF (Linker et al., 2011), indicating that fumarates in vivo act, in part, through activation of the Nrf2 pathway. In line with these data, Nrf2 knockdown prevented the dose-dependent protective effects of fumarates on ROS-induced cell death in astrocytes and neurons (Scannevin et al., 2012).

Besides their potent antioxidant properties, fumarates display strong immunomodulatory and anti-inflammatory properties. Decreased numbers of circulating leukocytes and lymphocytes have been observed in Tecfidera™-treated MS patients (Gold et al., 2012; Fox et al., 2012). Moreover, fumarates induce a shift from a Th1 towards a Th2 cytokine profile (de Jong et al., 1996; Ockenfels et al., 1998; Zoghi et al., 2011) and promote T cell apoptosis (Treumer et al., 2003; Spencer et al., 2015). Importantly, DMF also interferes with transendothelial leukocyte migration by downregulation of key cellular adhesion molecules on human umbilical vein endothelial cells (Wallbrecht et al., 2011; Vandermeeren et al., 1997). These findings were supported by the protective effects of fumarates in the EAE animal model, in which decreased T-cell and macrophage numbers were observed in the spinal cord of DMF-treated mice (Schilling et al., 2006). Furthermore, DMF also diminished the release of pro-inflammatory cytokines from microglia and astrocytes (Wilms et al., 2010). It is conceivable that the inhibitory spectrum of fumarates on cytokine-inducible genes and adhesion molecules is partly attributable to activation of the nuclear factor-κB (NF-κB) pathway (May and Ghosh, 1998). Moreover, the role of p62/SQSTM1, a scaffolding protein that has been linked to both Nrf2 and NF-κB signaling, is still unclear in the anti-inflammatory effects of fumarates. Although it has been demonstrated that DMF modulates cellular adhesion molecules involved in leukocyte migration, relatively little is known about the beneficial effects of fumarates at the level of the BBB. Hence, the aim of the current study is to elucidate the potential therapeutic effects of fumarates on inflammatory-mediated changes in BBB function and gain insight into the underlying mechanism.

Materials and methods

Cell culture

The human brain endothelial cell line hCMEC/D3 (Weksler et al., 2005) was provided by Dr. P.-O. Couraud (Institut Cochin, Universite Paris Descartes, Paris, France) and grown in endothelial cell basal medium-2 supplemented with human EGF, hydrocortisone, GA-1000, FBS, VEGF, human fibroblast growth factor (FGF)-B, R3-IGF-1, ascorbic acid, and 2.5% fetal calf serum (Lonza). The immortalized human brain endothelial cell line hCMEC/D3 was cultured as described previously (Weksler et al., 2005).

Monocyte adhesion assay

Monocyte adhesion was determined as described previously (van der Goes et al., 2001; Mizee et al., 2014). hCMEC/D3 cells were cultured as confluent monolayers in 96-well plates (Corning) and pre-treated with indicated non-toxic concentrations of MMF or vehicle control (DMSO) in the presence of 5 ng/ml TNF for 24 h. Unstimulated cells served as controls. Human primary monocytes were isolated from fresh buffy coats (Sanquin, Amsterdam, The Netherlands) by standard density centrifugation over Ficoll (Amersham Biosciences) followed by MACS sorting of peripheral blood mononuclear cells (PBMCs) with anti-CD14 MicroBeads (Miltenyi Biotec). Monocytes were fluorescently labeled with 0.5 μM calcein-AM (Molecular Probes, Eugene, OR, USA), washed twice with cold medium and suspended in RPMI + 0.5% BSA + 25 mM Hepes to a final concentration of 2 × 10^6 cells. A standard curve was made from the monocyte cell suspension and BECs were washed twice with pre-warmed medium prior to adding the monocytes to the BEC monolayers. Monocytes were allowed to adhere for 1 h at 37 °C and in 5% CO_2. Non-adherent cells were removed by washing twice with pre-warmed medium and lysed with 0.1 M NaOH. Fluorescence intensity was measured (FLUOstar Galaxy, BMG Lab technologies, Offenburg, Germany; excitation 480 nm, emission 520 nm) and the number of adhered monocytes quantified using a calibration curve as described previously (van der Goes et al., 2001; de Vries et al., 2002).

Monocyte migration

Monocyte transendothelial migration was analyzed by growing hCMEC/D3 cells in a confluent monolayer in 96-wells plates and pretreating them with indicated concentrations of MMF or DMSO in the presence of 5 ng/ml TNF for 24 h. Monocytes (6 × 10^4/well) were added to the plates and allowed to settle and migrate for a 4 h period. To monitor monocyte migration, co-cultures were placed in an inverted phase-contrast microscope (Nikon Eclipse TE300) equipped with a temperature-controlled (37 °C), 5% CO_2 gassed chamber as described previously (van der Goes et al., 2001). A field (220 × 220 μm) was randomly selected and recorded for 10 min at 50 times normal speed by using a color video 3CCD camera (Sony, with a CMAD2 adapter) coupled to a time-lapse video recorder (Sony SVT S3050P). Movies were analyzed by counting the number of cells that had migrated through the monolayer (visible as motile cells with phase-dark appearance) and the number of cells left on top of the endothelial cells (phase-bright appearance) as described before (Reijerkerk et al., 2008; Reijerkerk et al., 2012). The migratory capacity was calculated as the number of migrated cells expressed as a percentage of total cells within each field.

Flow cytometric analysis

Flow cytometric analysis of vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1) expression was performed using BECs incubated with or without MMF and 5 ng/ml TNF for 24 h. Cells were detached from 24-well culture plates (Corning) by collagenase type I treatment (1 mg/ml, Sigma). Cells were incubated with monoclonal mouse anti-ICAM-1 (Rek-1, 5 μg/ml, a kind gift from the Department of Tumor Immunology, University Medical Center, St. Radboud, Nijmegen, The Netherlands) or mouse anti-VCAM (AbD Serotec, UK) for 30 min at 4 °C. Binding was detected using FITC-conjugated goat-anti-mouse antibodies (1 μg/ml; Invitrogen, Carlsbad, CA, USA). Omission of primary antibodies served as a negative control. Fluorescence intensity was

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