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BRAIN RESEARCH

Research Report

Cytokine signaling in the human brain capillary endothelial cell line hCMEC/D3

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ABSTRACT

Brain microvascular endothelial cells are part of the blood–brain barrier and participate actively in immunological processes including cytokine-mediated inflammatory reactions. Using the human brain capillary endothelial cell line hCMEC/D3, activation of JAK/STAT signaling pathways were studied in response to stimulation by cytokines. The phenotype of hCMEC/D3 cells was confirmed by flow cytometry analysis of cell adhesion factors (cluster of differentiation molecules CD31 and CD34) and the von Willebrand factor endothelial marker was detected by immunofluorescence. Strong STAT1, STAT6 and STAT3 activation was observed in response to interferon-gamma (IFN-γ), interleukin 4 (IL-4) and interleukin 6 (IL-6), respectively. Nuclear translocation of phosphorylated STAT proteins was visualized by confocal microscopy. Treatment of hCMEC/D3 cells with IFN-γ resulted in interferon-induced upregulation of major histocompatibility complex (MHC) class I within 48 h. Interferon-alpha (IFN-α) did not activate STAT1 or STAT3 nor did it induce MHC class I upregulation. Therefore, hCMEC/D3 cells were judged to be non-responsive to IFN-α. We also observed that hCMEC/D3 cells exhibit functional expression of alternative cytokine signal transduction pathways (i.e. TNF-α mediated activation of NF-κB). Together these results indicate that human blood–brain barrier hCMEC/D3 cells are responsive towards stimulation with various cytokines. We conclude that this unique cell line can be used to explore in vitro human blood–brain barrier functionality under proinflammatory conditions.

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Abbreviations: hCMEC/D3, brain capillary endothelial cell line; JAK, Janus Tyrosine Kinase; NF-κB, nuclear factor “kappa-light-chain-enhancer” of activated B-cells; STAT, Signal Transducers and Activators of Transcription; phospho STAT, phosphorylated STAT; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; BBB, blood–brain barrier

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1. Introduction

The blood–brain barrier (BBB) is a cellular barrier, which is located at the interface between the blood and the central nervous system (CNS). The anatomical basis of the BBB is a single layer of endothelial cells lining the inner surface of capillaries in the brain. Due to the restrictive permeability and highly selective transport properties of these cells, the passage of solutes between the periphery and brain is tightly regulated. Functions of these cells include the regulation of ion balance, the facilitated and active transport of nutrients and the exclusion of potentially harmful substances from the CNS.

Besides these important transport and protective functions, the BBB has been proposed to modulate immune responses in the central nervous system (Pachter et al., 2003). As demonstrated using in vitro models of the BBB, brain microvascular endothelial cells express MHC (major histocompatibility complex) class I (Horwitz et al., 1999) and inducible class II (Male and Pryce, 1988) molecules (a prerequisite for antigen presentation by antigen presenting cells to T cells), produce inflammatory cytokines (Verma et al., 2006; Vanier et al., 2009; Chakraborty et al., 2010) such as interleukin (IL)-1α, IL-6, IL-8, IL-10 and tumor necrosis factor α (TNF-α) and regulate transmigration of leukocytes across brain endothelia (Persidsky, 1999; Man et al., 2007; Rampon et al., 2008). In addition, the BBB is highly responsive towards stimulation by proinflammatory cytokines, as encountered under pathological conditions like cerebral inflammation (Abbott, 2000; de Boer and Gaillard, 2006). In cell culture models, cytokines such as TNF-α, IL-6 and IL-1β were shown to increase the paracellular permeability of the BBB in a dose- and time-dependent manner (de Vries et al., 1996; Förster et al., 2008) whereas type 1 interferons (IFN-α,β) had opposite effects (Gillies and Su, 1995; Kraus et al., 2004). Associated changes in solute penetration rates across the brain endothelium have been attributed to effects including modulation of tight junction integrity (Hawkins and Davis, 2005) or expression levels of drug transporting ATPases such as BCRP (ABCG2) and P-glycoprotein (ABCB1) (Poller et al., 2010).

Neurodegenerative disorders such as multiple sclerosis (Popko et al., 1997), Alzheimer's disease (Maccioni et al., 2009), Parkinson's disease (Nagatsu and Sawada, 2005) and other acute cerebral conditions (e.g. ischemic insult (Tuttolomondo et al., 2008) and traumatic brain injury) are characterized by neuroinflammatory processes. In these cases it has been recognized that blood–brain barrier integrity is compromised, possibly leading to progression or even suggesting that blood–brain barrier malfunction contributes to the etiology of such pathologies (for a review see (Zlokovic, 2008)). Local proinflammatory cytokines and chemokines may thereby modulate a plethora of BBB functions, which has stimulated an increasing interest in the interplay between brain microvascular endothelial cells and these mediators. Previously, Weksler et al. (2005) established an in vitro model of the human BBB (hCMEC/D3) by transducing primary human brain endothelial cells by lentiviral vectors incorporating human telomerase and SV40 T antigen. This model was used to study active, passive and bidirectional drug transport (Poller et al., 2008).

It was the aim of the present work to study the interaction of cytokines with hCMEC/D3 cells. In view of the importance of these processes for BBB functionality (e.g. cellular permeability, integrity or signal transduction), the presence and functionality of the JAK/STAT pathway (Janus Tyrosine Kinase/Signal Transducers and Activators of Transcription) (Pestka et al., 2004) was investigated upon cytokine stimulation with IL-4, IL-6, INF-α and IFN-γ. This intracellular signal transduction pathway is involved in regulation and immune-defense. Binding of ligands to their transmembrane receptors leads to phosphorylation and thus activation of members of the intracellular JAK protein family. The activated receptor-kinase complexes recruit members of the STAT proteins, which then translocate to the nucleus in a phosphorylated and dimerized state where they subsequently promote transcription.

2. Results

2.1. *Expression of endothelial markers*

The blood–brain barrier (BBB) hCMEC/D3 cell line was recently established and validated for drug transport studies (Weksler et al., 2005; Poller et al., 2008). We first confirmed the human endothelial phenotype of the hCMEC/D3 cell line. As demonstrated by confocal microscopy studies (Fig. 1), hCMEC/D3 cells express von Willebrand factor. Moreover, flow cytometry analysis revealed expression of cluster of differentiation surface antigens CD31 and CD34, which are markers of endothelial cells (Fig. 2A and B).

2.2. *Activation of JAK/STAT signal transduction pathways*

hCMEC/D3 cells were examined for JAK/STAT activation following incubation with various cytokines (IFN-α, IFNγ, IL-4 and IL-6). Cells were fixed, permeabilized and subject to flow cytometry with intracellular phosphorylated STATs detected using anti-phospho-STAT1, STAT3 or STAT6 antibodies (Fig. 3). A strong anti-phospho-STAT1 shift was observed in response to INF-γ (Fig. 3A, black histogram) as compared to isotype matched negative controls (white histograms) or untreated cells (grey

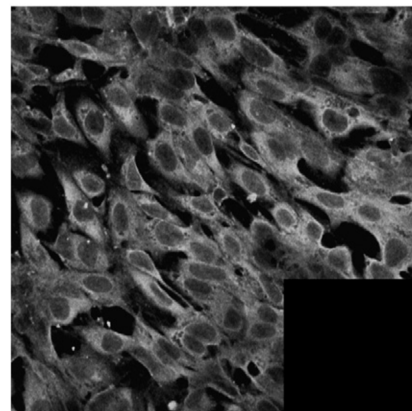

Fig. 1 – Expression of von Willebrand factor by hCMEC/D3 cells. Confocal microscopy analysis using polyclonal anti-von Willebrand antibody. Secondary antibody: Cy2-labeled goat anti-rabbit antibody. Insert: negative control using secondary antibody only. Magnification: 40×.

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