ELSEVIER

Nanomedicine: Nanotechnology, Biology, and Medicine
xx (2017) xxx–xxx

nanomedicine
Nanotechnology, Biology, and Medicine

nanomedjournal.com

Inflammation-induced brain endothelial activation leads to uptake of electrostatically stabilized iron oxide nanoparticles *via* sulfated glycosaminoglycans

Dominique Berndt[a,e], Jason M. Millward[a,b], Jörg Schnorr[c], Matthias Taupitz[c], Verena Stangl[d], Friedemann Paul[b,e], Susanne Wagner[c], Jens T. Wuerfel[e], Ingolf Sack[c], Antje Ludwig[d], Carmen Infante-Duarte[a,b,*]

[a]*Institute for Medical Immunology, Charité-Universtitätsmedizin Berlin, Berlin, Germany*
[b]*Experimental and clinical Research Center, Max Delbrueck Center for Molecular Medicine and Charité Universitätsmedizin, Berlin*
[c]*Department of Radiology, Charité-Universitätsmedizin Berlin, Berlin, Germany*
[d]*Medizinische Klinik mit Schwerpunkt Kardiologie und Angiologie, Charité, Universitätsmedizin Berlin, Berlin, Germany*
[e]*Cluster of Excellence NeuroCure and Department of Neurology and Experimental and Clinical Research Center, Universitätsmedizin Berlin, Berlin, Germany*

Received 12 August 2016; revised 20 December 2016; accepted 12 January 2017

Abstract

Based on our previous data on the presence of very small superparamagnetic iron oxide nanoparticles (VSOP) on brain endothelial structures during experimental autoimmune encephalomyelitis (EAE), we investigated the mechanisms of VSOP binding on inflamed brain endothelial cells *in vivo* and *in vitro*. After intravenous application, VSOP were detected in brain endothelial cells of EAE animals at peak disease and prior to clinical onset. *In vitro*, inflammatory stimuli increased VSOP uptake by brain endothelial bEnd.3 cells, which we confirmed in primary endothelial cells and in bEnd.3 cells cultured under shear stress. Transmission electron microscopy and blocking experiments revealed that during inflammation VSOP were endocytosed by bEnd.3. Modified sulfated glycosaminoglycans (GAG) on inflamed brain endothelial cells were the primary binding site for VSOP, as GAG degradation and inhibition of GAG sulfation reduced VSOP uptake. Thus, VSOP-based MRI is sensitive to visualize early neuroinflammatory processes such as GAG modifications on brain endothelial cells.

Key words: Nanoparticles; Brain endothelial cells; Neuroinflammation; Glycosaminglycans; Extracellular matrix; VSOP

The migration of cells and soluble factors from the periphery into the central nervous system (CNS) is strictly regulated by the blood–brain barrier (BBB). The BBB is a complex structure, composed of the brain vascular endothelial cells, pericytes, and astroglia.[1] In addition, elements of the extracellular matrix (ECM) play an essential role in maintaining BBB integrity, forming the parenchymal and the endothelial basement membranes.[2] Moreover, in contrast to the vascular endothelia in the periphery, the endothelial cells of the CNS vasculature are connected by tight junctions (TJ) and are not fenestrated.

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; GAG, glycosaminglycans; MRI, magnetic resonance imaging; MS, multiple sclerosis; PLP, proteolipid protein; SPIO, superparamagnetic iron oxide; TJ, tight junctions; VSOP, very small superparamagnetic iron oxide nanoparticles.

Funding: This work was supported by the German Research Foundation (Deutsche Forschungs-gemeinschaft, DFG), within the Clinical Research Unit KFO 213, projects IN 156/1-1, WU713/1-1 and TA 166/3-2 as well as Exc.257 to F. Paul.

Conflicts of interests: The authors have no conflict of interests

*Corresponding author at: Experimental Neuroimmunology, Charité-Universitätsmedizin Berlin, Institute for Medical Immunology, Campus Virchow Klinikum, Berlin, Germany.
E-mail address: carmen.infante@charite.de (C. Infante-Duarte).

http://dx.doi.org/10.1016/j.nano.2017.01.010
1549-9634/

During inflammatory diseases of the CNS such as multiple sclerosis (MS), the integrity of the BBB becomes compromised. Studies of the animal model of MS, experimental autoimmune encephalomyelitis (EAE) have shown that the CNS endothelium undergoes alterations during neuroinflammation, including the disruption of TJ, leading to increase permeability.[3] Increased vascular permeability associated with active inflammation can be visualized *in vivo* by gadolinium contrast enhanced magnetic resonance imaging (MRI).[4] However, in both human disease and the EAE model, there are discrepancies between the disease activity measured by gadolinium-enhancing lesions and the degree of clinical disability,[5–7] pointing to the limitations of the established contrast-agent-based MRI to visualize inflammatory lesions.

Superparamagnetic iron oxide (SPIO) nanoparticle-enhanced MRI holds promise for neuro-inflammation sensitive imaging. SPIO and ultra-small SPIO (USPIO) have been shown to be readily taken up by phagocytes and have been used to identify infiltrating macrophages in EAE brains.[8–10] Very small superparamagnetic iron oxide nanoparticles (VSOP) are smaller than other iron oxide nanoparticles, with a hydrodynamic diameter of ~7 nm, achieved through electrostatic stabilization with citrate.[11,12] The excellent long-term stability of our citrate-coated VSOP under physiological conditions has been demonstrated in previous pre-clinical and clinical studies.[12–16] In addition, in a previous study, we showed that VSOP could detect EAE lesions that were not detectable with a conventional gadolinium based contrast agent.[17] Further, we demonstrated that VSOP could highlight the early involvement of the choroid plexus in EAE.[18] In that study, we also detected the presence of VSOP on CNS endothelial structures, both in inflammatory lesions in the parenchyma and in the choroid plexus.[18] However, it remains unsolved if the particles are able to bind to or penetrate directly to the brain endothelial cells during inflammation or are rather present inside endothelium-associated phagocytes. Here we confirm the presence of VSOP on/in inflamed endothelial cells *in vivo* and used an *in vitro* model to prove the direct binding of VSOP to endothelial cells and to investigate mechanisms of interaction between the particles and the endothelial barrier during inflammation.

Methods

Induction of EAE

All procedures were performed in accordance with protocols approved by the local animal welfare committee (LAGeSo) in conformity with national and international guidelines to minimize discomfort to animals (86/609/EEC). Female SJL/J mice were purchased from Janvier and housed under standard conditions. Passive EAE was induced, as described previously.[11] In brief, donor mice were immunized with proteolipid protein peptide 139–151 ($PLP_{139–151}$), along with Complete Freund's adjuvant. Ten days later, draining lymph nodes were collected and lymphocytes isolated. Lymphocytes were then re-stimulated *in vitro* for 4 days with 12.5 μg/ml $PLP_{139–151}$ to generate encephalitogenic lymphocytes as described.[18] Recipient mice received 5×10^6 encephalitogenic lymphocyte blasts injected intravenously. The recipient mice were monitored daily, and assigned a clinical score as follows: 0, no disease; 1, tail weakness; 2, paraparesis; 3, paraplegia; 4, paraplegia with forelimb weakness; 5, moribund or dead animals. These animals were killed at peak disease. In a separate cohort of 10 mice, active EAE was induced by PLP immunization; this cohort was killed prior to onset of clinical signs, at day 6 post-immunization.

VSOP, histology

Very Small **S**uperparamagnetic Iron **O**xide **P**articles VSOP were produced at the Charité Department of Radiology as described previously described[19] (preparation batch 170,712) with the following properties: iron concentration of 103.9 mmol/l, citrate content 3.534 g/l, yielding a 10.7% weight ratio of citric acid to iron; $R_1 = 26.61$, $R_2 = 70.33$; size distribution 6.5–10.1; Z-average of 14.2 and a zeta potential of −17.0 mV (1 mM VSOP measured in 10 mM NaCl solution). Mice with EAE were administered 0.2 mmol/kg VSOP i.v. Twenty-four hours later, the mice were terminally anesthetized, then transcardially perfused with 20 ml PBS, then with 20 ml zinc fixation solution (0.5% zinc acetate, 0.5% zinc chloride, 0.05% calcium acetate).[20] The brains were extracted and postfixed in zinc solution for 3 days at room temperature. The tissues were cryoprotected overnight in 30% sucrose, frozen and cut into 12 μm sections. Prussian Blue staining for iron detection was done using Perl's method (incubation with 1% potassium hexacyanoferrate and 1% HCl), followed by counterstaining with Nuclear Fast Red.

Cell culture conditions and treatment with VSOP

Murine brain vascular endothelial bENd.3 cells (ATCC, USA) were cultured on 6-well plates at a density of 10^5 cells per well in DMEM supplemented with 10% FCS, at 37 °C and 5% CO_2. bEnd.3 cells were cultured in serum-free medium 24 h prior to the application of VSOP in the presence or absence of TNF-alpha at a concentration of 10 ng/ml. For enzyme digestion of glycosaminoglycans (GAG), bEnd.3 cells were incubated with either 1.2 mU chondroitinase ABC, 0.4 mU heparinase III, 30 U hyaluronidase or a mixture of all three enzymes for 3 h prior to treatment with the VSOP.

For blocking endocytosis, bENd.3 cells were incubated under standard conditions following activation with 10 ng/ml TNF-alpha for 24 h. Cells were then incubated in DMEM supplemented with either 10 μM monensin for 2 h or 0.4 M Sucrose for 30 min prior to incubation with the VSOP. After that, Prussian Blue staining or iron quantification was performed.

For blocking sulfation, bEnd.3 cells were incubated in a medium enriched with 50 mM sodium chlorate for a duration of 48 hours prior to following activation steps and incubation with VSOP.

Transmission electron microscopy

For electron microscopy, bENd.3 cells were treated for 5 minutes or 2 hours with VSOP, then washed twice with PBS and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer solution overnight at 4 °C. After several washes with 0.1 M cacodylate buffer, the cells were post-fixed in 1% osmium tetroxide/0.8% $K_4[Fe(CN)_6]$ in 0.1 M cacodylate buffer for 1.5 hours and washed 2 × 10 min. in PBS. The cells were