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IN VIVO ANALYSIS OF NEUROINFLAMMATION IN THE LATE CHRONIC PHASE AFTER EXPERIMENTAL STROKE

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Abstract—*Background and purpose: In vivo* imaging of inflammatory processes is a valuable tool in stroke research. We here investigated the combination of two imaging modalities in the chronic phase after cerebral ischemia: magnetic resonance imaging (MRI) using intravenously applied ultra small supraparamagnetic iron oxide particles (USPIO), and positron emission tomography (PET) with the tracer [^{11}C]PK11195. *Methods:* Rats were subjected to permanent middle cerebral artery occlusion (pMCAO) by the macrosphere model and monitored by MRI and PET for 28 or 56 days, followed by immunohistochemical endpoint analysis. To our knowledge, this is the first study providing USPIO-MRI data in the chronic phase up to 8 weeks after stroke. *Results:* Phagocytes with internalized USPIOs induced MRI-T2* signal alterations in the brain. Combined analysis with [^{11}C]PK11195-PET allowed quantification of phagocytic activity and other neuroinflammatory processes. From 4 weeks after induction of ischemia, inflammation was dominated by phagocytes. Immunohistochemistry revealed colocalization of Iba1+ microglia with [^{11}C]PK11195 and ED1/CD68 with USPIOs. USPIO-related iron was distinguished from alternatively deposited iron by assessing MRI before and after USPIO application. Tissue affected by non-phagocytic inflammation during the first week mostly remained in a viably vital but remodeled state after 4 or 8 weeks, while phagocytic activity was associated with severe injury and necrosis accordingly. *Conclusions:* We conclude that the combined approach of USPIO-MRI and [^{11}C]PK11195-PET allows to observe post-stroke inflammatory processes in the living animal in an intraindividual and longitudinal fashion, predicting long-term tissue fate. The non-invasive imaging methods do not affect the immune system and have been applied to human subjects before. Translation into clinical applications is therefore feasible.

Key words: chronic post-stroke phase, *in vivo* imaging, PET, MRI, phagocytic activity, regional tissue fate.

INTRODUCTION

Cerebral ischemia is accompanied by various cellular and molecular processes, which contribute to restoration of brain function after stroke (Wieloch and Nikolich, 2006; Murphy and Corbett, 2009). Especially inflammation impacts on neuroplasticity and long-term recovery (del Zoppo, 2010; Morrison and Filosa, 2013; Ruscher et al., 2013). *In vivo* visualization of inflammatory reactions may facilitate translation of novel therapies into clinical studies (Fumagalli et al., 2013; Heiss, 2014; Quattromani et al., 2014). Among others, magnetic resonance imaging (MRI) using ultra small supraparamagnetic iron oxide particles (USPIO) (Deddens et al., 2012; Marinescu et al., 2013) and positron emission tomography (PET) with the radiotracer [^{11}C]PK11195 (Schroeter et al., 2009; Jacobs et al., 2012) represent attractive approaches.

The lipophilic tracer [^{11}C]PK11195 binds to the 18-kDa translocator protein (TSPO), a cholesterol-transporter found on the membrane of mitochondria (Papadopoulos et al., 2006). After brain injury, its expression on microglia, astrocytes and macrophages increases, representing a target for imaging (Stephenson et al., 1995; Chen and Guilarte, 2008). Unfortunately the method is limited due to short half-life of ^{11}C and low signal-to-noise ratio (Jucaite et al., 2012; Dickens et al., 2014). Moreover radiosynthesis of [^{11}C]PK11195 is very time consuming and expensive.

In MRI, intravenously applied USPIOs lead to hypointense T2*-signal changes in the lesioned central nervous system (CNS) by invasion of USPIO-loaded macrophages (Nighoghossian et al., 2007; Desestret et al., 2013). This was shown to occur independently of a potentially associated blood–brain barrier breakdown (Stoll and Bendszus, 2010; Yang et al., 2013). Translational approaches demonstrated USPIO+ phagocytes also in the human CNS after stroke (Saleh et al.,

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Abbreviations: CNS, central nervous system; MRI, magnetic resonance imaging; PET, positron emission tomography; pMCAO, permanent middle cerebral artery occlusion; USPIO, ultra small supraparamagnetic iron oxide particles; VOI, volumes of interest.

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2004, 2007). However, these findings have been questioned by studies using transient stroke models in high-field MRI (Desestret et al., 2009; Farr et al., 2011; Harms et al., 2013). In addition dysregulation of natural neuroinflammatory responses by USPIOs has been discussed (Siglienti et al., 2006; Hsiao et al., 2008). Recent results furthermore suggest that iron deposition naturally occurs after stroke (Danielisova et al., 2004; Li et al., 2009; Hagemeier et al., 2012) and other diseases associated with persistent microglia activation (Zivadinov et al., 2011).

The present study was conducted to evaluate the validity of combined imaging by USPIO-MRI and [^{11}C]PK11195-PET as a method to longitudinally and intraindividually analyze stroke-induced inflammatory processes in the living organism.

EXPERIMENTAL PROCEDURES

Experimental design

Thirteen male Wistar rats underwent permanent middle cerebral artery occlusion (pMCAO) by the macrosphere model, which closely resembles the dynamic patterns of neuroinflammatory procedures in human stroke (Gerriets et al., 2003; Walberer et al., 2010). At day 6 (d6), d27, and d55, animals were subjected to both MRI (T2, T2*) and PET, using the tracer [^{11}C]PK11195 to investigate neuroinflammatory processes. Directly afterward, USPIOs (300 µmol Fe/kg) were injected intravenously (iv) followed by another MRI 24 h later, i.e., at d7, d28 and d56, to analyze USPIO-associated signal-changes. After the first MRI at d6, three rats were excluded from the study due to significantly smaller infarcts without cortical involvement. From the remaining 10 animals five were sacrificed at d28 and five at d56 for endpoint analyses by immunohistochemistry (Fig. 1A). Animals were not perfused and therefore provided fresh frozen tissue.

Another group of 13 male Wistar rats served as a control for stroke-associated iron-deposition. They underwent pMCAO but were not subjected to USPIOs. At d3 (n = 2), d7 (n = 2), d14 (n = 3), d28 (n = 3) and d56 (n = 3) immunohistochemical analyses were performed.

Animals and surgery

Animal procedures were in accordance with the German Laws for Animal Protection and approved by the local animal care committee and governmental authorities. Rats (320–365 g) were anesthetized with isoflurane and nitrous oxide/oxygen (65%/35%) and additionally received Carprofen (Rimadyl®; 5 mg/kg/day s.c. for 3 days) for surgery. Body temperature and respiration rate were monitored by combination of in-house equipment and DASYLab 9.0 (DasyLab, Moenchengladbach, Germany).

MRI and PET

T2- and T2*-weighted MRI was performed on a 4.7 T BioSpec system (Bruker BioSpin, Ettlingen, Germany), PET imaging on a microPET Focus 220 scanner (Concorde Microsystems, Inc., Knoxville, TN, USA; 63 image planes; 1.5-mm full width at half maximum). The radiolabelled tracer [^{11}C]PK11195 was produced as described elsewhere in more detail (Hamacher et al., 1986; Shah et al., 1994; Schroeter et al., 2009). Binding potential of [^{11}C]PK11195 was calculated by the simplified reference tissue model (SRTM) using the contralateral hemisphere as reference region (Lammertsma and Hume, 1996).

Image analysis and volume quantification

To differentiate between phagocytic activity and other neuroinflammatory processes in the brain, we co-registered PET- and MR-images using the software VINCI (Cizek et al., 2004). Specific volumes of interest (VOIs, mm^3) were defined: VOI(USPIO), VOI(USPIO + PK), VOI(PK) and VOI(CNS) (Fig. 1B–D, Table 1). MR-images were normalized in gray-scale intensity. Accumulation of iron induced focal hypointense T2* signal changes. For precise identification of USPIOs, the T2*-image acquired 24 h after was digitally subtracted from a T2*-image acquired before USPIO administration in each animal (Fig. 1B). Here hyperintense areas represented specifically USPIO + tissue, excluding any other sources of iron deposition that had been present before this effective USPIO administration including potential remnants of former USPIO injections on d28 and d56. The corresponding VOI(USPIO) was reconfirmed on the hypointense signal of T2*-MRI after USPIO application (Fig. 1B, Table 1) and denominated the area of phagocytic activity after stroke. Threshold for [^{11}C]PK11195 associated VOI-definition was 50% binding capacity of the tracer. A VOI was thus accounted positive if it appeared in green or hotter color. Fusing VOI(USPIO) on the [^{11}C]PK11195-signal allowed to define colocalization of USPIOs and [^{11}C]PK11195 leading to VOI(USPIO + P K) (Fig. 1C, Table 1). The volume [^{11}C]PK11195 was also quantified representing VOI(PK) (Fig. 1D, Table 1). The volume resulting from subtraction of VOI(USPIO + PK) from VOI(PK) was regarded as non-phagocytic neuroinflammation, namely VOI(CNS) (Fig. 1D, Table 1).

Immunohistochemistry

Fresh frozen slices (10 µm) were stained with Hematoxylin and Eosin (H&E), Anti-Iba1 (dilution 1:1000, Wako, Neuss, Germany, cat-# 019-19741) and ED1/CD68 (dilution 1:4000, AbD Serotec, Oxford, UK, cat-# MCA341) both using the ABC-Kit (Vector Laboratories, Burlingame, CA, USA). The Prussian blue reaction enhanced by diaminobenzidine detected iron (Schroeter et al., 2004). In each staining result, the hemisphere contralateral to ischemia served as a negative control.

Statistical analysis

For statistical analysis we used Excel® (Microsoft Office Excel 2003) and SPSS-Statistics® (IBM SPSS-Statistics 22). VOIs were defined in mm^3 providing parametric data. Student's t-test was performed in paired groups, results were defined significant at $p < 0.05$, confidence