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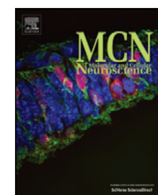

Early monitoring and quantitative evaluation of macrophage infiltration after experimental traumatic brain injury: A magnetic resonance imaging and flow cytometric analysis

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ABSTRACT

The inflammatory response following traumatic brain injury (TBI) is regulated by phagocytic cells. These cells comprising resident microglia and infiltrating macrophages play a pivotal role in the interface between early detrimental and delayed beneficial effects of inflammation. The aim of the present study was to monitor the early effect of monocyte/phagocytic accumulation and further to explore its kinetics in TBI mice. Localized macrophage population was monitored using ultrasmall superparamagnetic iron oxide (USPIO) nanoparticle enhanced *in vivo* serial magnetic resonance imaging (MRI). Flow cytometry based gating study was performed to discriminate between resident microglia (Ly6G^{-}CD11b^{+}CD45^{low}) and infiltrating macrophages (Ly6G^{-}CD11b^{+}CD45^{high}) at the injury site. The T_2* relaxation analysis revealed that maximum macrophage infiltration occurs between 66 and 72 h post injury (42–48 h post administration of USPIO) at the site of inflammation. This imaging data was well supported by iron oxide specific Prussian blue staining and macrophage specific F4/80 immunohistochemistry (IHC) analysis. Quantitative real-time PCR analysis found significant expression of monocyte chemoattractant protein-1 (MCP-1) at 72 h post injury. Also, we found that flow cytometric analysis demonstrated a 7-fold increase in infiltrating macrophages around 72 h post injuries as compared to control. The MR imaging in combination with flow cytometric analysis enabled the dynamic measurement of macrophage infiltration at the injury site. This study may help in setting an optimal time window to intervene and prevent damage due to inflammation and to increase the therapeutic efficacy.

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1. Introduction

Neuroinflammation in CNS is an important mechanism in several types of neurological diseases such as traumatic brain injury (TBI), cerebral hemorrhage, cerebral infarction, stroke, and multiple sclerosis (Foley et al., 2009; Price et al., 2003; Saleh et al., 2004; Dousset et al., 2006). Traumatic brain injury comprises a broad range of physical, cognitive, behavioral, and emotional impairments that depend on the type, severity, and site of the injury (Weissenberger and Siren, 2010). Primary injury caused by direct impact to the head results in cortical contusion, vascular injury, axonal injury, hemorrhages, ischemia and inflammatory response. Secondary injury results from a cascade of cellular, vascular and biochemical pathophysiological events after primary injury (McIntosh et al., 1996; Danton and Dietrich, 2003). Brain inflammation is an important pathological mechanism in a sub-acute time window after primary TBI. Some cell types such as neutrophils, astrocytes, resident microglia/macrophages, as well as hematogenous macrophages, are activated after primary injury (Schoettle et al., 1990; Soares et al., 1995).

Inflammatory neurodegeneration is a condition of neuronal death caused by inflammatory responses. The active macrophages have been proposed to secrete large numbers of pro-inflammatory cytokines, nitric oxide, and oxygen free radicals and proceed in a process such as energy depletion, protein aggregation, and excitotoxicity which contribute to neuronal loss or dysfunction. Neuroinflammation can damage the

Abbreviations: ADC, apparent diffusion coefficient; ANOVA, analysis of variance; BBB, blood-brain barrier; DTI, diffusion tensor images; FA, fractional anisotropy; FOV, field of view; IHC, immunohistochemistry; ISA, imaging sequence analysis; MCP-1, monocyte chemoattractant protein-1; MGE, multi-gradient echo; MRI, magnetic resonance imaging; mRNA, messenger RNA; MSME, multi-slice multi-echo; PCR, polymerase chain reaction; qPCR, quantitative PCR; ROI, region of interest; R_2, relaxation rate; T_2, relaxation time; r_2, relaxivity; TBI, traumatic brain injury; TR, repetition time; TE, echo time; USPIO, ultrasmall superparamagnetic iron oxide.

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brain by a variety of mechanisms such as vascular wall inflammation, damage in blood-brain-barrier (BBB), the formation of edema, suppression of neurogenesis and activation of microglia (Gensel and Zhang, 2015; Morganti-Kossmann et al., 2002). Post-TBI events include activation of brain resident microglia and astrocytes as well as infiltration of activated leukocytes such as neutrophils, macrophages, and lymphocytes into the brain, which can increase neuronal damage (Habgood et al., 2007).

Inflammation may have beneficial and/or detrimental effects after TBI (Jin et al., 2010). The benefits are mainly due to secretion of neurotrophins, elimination of pathogens, clearing debris, and aiding repair, whereas the detrimental effects are probably the unintended release of neurotoxic substances that cause additional brain damage (Holmin et al., 1995). Recent research suggests that infiltrating macrophages may play a pivotal role at the interface between early detrimental and delayed beneficial effects of inflammation (Gensel and Zhang, 2015). Therefore, inhibition of infiltrating macrophages is considered as an attractive therapeutic strategy (Stoll et al., 1998). The contribution of activated resident microglia *versus* infiltrating macrophages to traumatic brain damage has been difficult to define because of similar immunophenotypes and other histological characteristics for these cells (Longbrake et al., 2007). The current standard for distinguishing between microglia and infiltrating macrophages is immunohistochemistry, which permits semi-quantitative analysis from localized regions. Gordon et al. described a column free magnetic separation method, whereas Bedi et al. have used CD11b immunomagnetic enrichment along with flow cytometry to isolate mouse microglia (Gordon et al., 2011; Bedi et al., 2013). However, it has been well established that the resident microglia along with neutrophils gets activated rapidly after an injury, but the blood-borne macrophages infiltrate the lesion site with a delay of at least 24 h (Schilling et al., 2003; Tanaka et al., 2003). By taking this point into consideration, here we describe a flow cytometry-based method to differentiate resident microglia and infiltrating macrophages after gating out activated neutrophils in a murine model of closed head traumatic brain injury.

Several published reports have shown a time frame of 1 day to 7 days for macrophage accumulation at the neuroinflammatory site. However, no one has so far precisely established the specific time window in which the iron oxide labeled macrophages home to the site of injury. Thus, the aim of the study that we undertook was to investigate the feasibility of phagocyte/macrophage labeling and its *in vivo* monitoring during the early stage after experimental TBI. We also explore the kinetics of phagocytic cells at the injury site and the discrimination between resident microglia and infiltrating macrophages by MR imaging and flow cytometry based gating analysis, respectively. Subsequently, to support MRI and flow cytometric findings, we also performed immunohistochemistry and mRNA analysis.

2. Materials and methods

2.1. Subjects and care of animals

All experimental procedures were conducted under the approval of the animal ethics committee of the Institute (Registration No. INM/IAEC/16/02, INMAS, Delhi); all procedures were carried out by the standard recommended manual on the use and care of laboratory animals. Ninety-two adult healthy Balb/c male mice (25–30 g, 6–8 weeks old) were taken for the experiment. They were given laboratory chow and water *ad libitum* and maintained under temperature-controlled conditions (25 ± 2 °C) with 12 h light/dark cycles.

2.2. Experimental design

Out of 92 mice taken for study, 32 mice were randomly selected for MRI. In the remaining 60 mice, 20 mice were used for histological investigation, 20 mice were used for mRNA analysis, and the other 20 mice were taken for flow cytometry analysis.

Mice selected for MRI were further subdivided into four groups. Mice in group-1 (n = 8) were injected with 0.9% NaCl after TBI, group-2 mice (n = 8) were injected with USPIO without any injury, group-3 & 4 mice (n = 8 each) were injected with USPIO after TBI. It has been reported that the resident microglia activates rapidly within 24 h after injury and the macrophage infiltration occurs at a later time (Schilling et al., 2003; Tanaka et al., 2003). To overcome the iron oxide accumulation of resident microglia and labeling of infiltrating macrophages, the USPIO was administered to each mouse after 24 h of injury. A serial *in vivo* MRI was carried out at respective time points in all the four groups.

Mice were sacrificed by cervical dislocation (n = 4/time point) at five-time points for all histological, gene expression analysis and flow cytometric investigation. The detail graphical setup is shown in Fig. 1 to explain the experimental design.

2.3. Traumatic brain injury model

Traumatic brain injury was induced in mice by modified Marmarou's weight-drop model as previously reported, which closely mimics the real-life injury (Mishra et al., 2016b; Marmarou et al., 1994). Mice were anesthetized with a cocktail of ketamine (80 mg/kg b/w) and xylazine (10 mg/kg b/w) and placed onto the stereotactic holder under the weight-drop device. A cylindrical brass of weight-35 g was freely dropped from a height of 40 cm on the sagittal midway of the mouse brain. Approximately a force of 0.137 N was induced to create injury at the same region and of similar intensity in all the study animals. After the injury, the animals were monitored for 30 min with supplemental O_2 and returned to their respective parental cages until MRI assessment. The occurrence of injury was confirmed in the MRI scanner after 6 h post injury in all the mice used for TBI.

2.4. Contrast agents

High transverse relaxivity magnetic USPIO was synthesized in our laboratory by modified alkaline co-precipitation method as reported earlier (Mishra et al., 2015, 2016a). Contrast agent was administered to mice *via* tail as an intravenous bolus dose of 3.0 mg Fe/kg body weight. The mice used in groups 2, 3, and 4 and other 60 mice utilized in histological, gene expression and flow cytometric analysis were injected with contrast agent.

2.5. MRI acquisition and processing

All MRI experiments were carried out on a 7 T horizontal bore animal MRI scanner (Bruker Biospec USR 70/30, AVANCE III) equipped with a BGA12S gradient system capable of producing 400 mT/m pulse gradients in each of the three orthogonal axes and interfaced to a Bruker Paravision 5.1 console. Anesthetized animals were placed in prone position on an animal bed and then slide into the center of the magnet bore. Radio frequency excitation was accomplished with 72 mm inner diameter linear birdcage coil and mouse brain array coil (single tuned 1H 2 × 2 array, four channels) was used for signal reception. The parameters were as follows:

T_2 maps were acquired using MSME_T_2 (multi-slice multi-echo) sequence (repetition time TR = 3500 ms, echo time TE = 13–208 ms with a total of 16 echoes and echo length of 13 ms, flip angle = 180°). T_2^* maps were acquired using MGE_T_2^* (multi-gradient echo) sequence (TR = 1500 ms, 12 echo TE/echo spacing = 4.50–65.45 ms/5.54 ms, matrix size = 256/192, flip angle = 30°). Diffusion-weighted images were acquired using diffusion tensor imaging sequence (TR/TE = 3800/31 ms, number of diffusion encoding directions = 46, b value = 670 s/mm^2, matrix size = 128 × 128). Apparent diffusion coefficient (ADC) maps and fractional anisotropy (FA) maps were obtained

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