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Full-length Article

High-dosage granulocyte colony stimulating factor treatment alters monocyte trafficking to the brain after experimental stroke

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ABSTRACT

Ischemic stroke elicits a prompt inflammatory response that is characterized by a well-timed recruitment of peripheral immune cells to the brain. Among these, monocytes play a particularly important, but multifaceted role and have been increasingly recognized to affect stroke outcome. Granulocyte colony stimulating factor (GCSF) is known for its immunosuppressive actions on mononuclear cells, but previous studies in the stroke field were mainly confined to its neuroprotective actions. Herein, we investigated whether GCSF affects post-stroke inflammation in a mouse model of focal brain ischemia by modulating monocyte responses. Treatment with GCSF was controlled by vehicle injection, sham surgery and naive animals. Despite a significant monocytosis, high-dosage GCSF reduced the number of brain-infiltrating monocytes/macrophages four days after stroke. Lower numbers of mononuclear phagocytes in the brain were associated with smaller cerebral edema and improved motor outcome after stroke. GCSF treatment over 72 h, but not 24 h diminished integrin expression on circulating Ly6C+ inflammatory monocytes. In vitro experiments further revealed that GCSF strongly promotes interleukin (IL)-10 secretion by activated mononuclear cells. Blockade of the IL-10 receptor partly reversed GCSF-induced downregulation of integrin surface expression. Overall, our results suggest that high-dosage GCSF mitigates monocyte infiltration after stroke, likely by attenuating integrin-mediated adhesion to the brain endothelium in an IL-10-dependent manner. Lower amounts of mononuclear cells in the brain translate to less severe brain edema and functional impairment and thus support a harmful role of Ly6C+ inflammatory monocytes in the acute stage of stroke.

1. Introduction

Cerebral stroke induces a prompt immune response in the damaged brain that is initially dominated by infiltrating polymorphonuclear neutrophils (PMN) which are subsequently replaced by monocytes and their descendants. Originally regarded as an epiphenomenon of ischemic injury, post-stroke inflammation is increasingly recognized to affect stroke outcome, either by causing collateral damage or by promoting clearance, wound healing and regeneration (Gliem et al., 2012; Hammond et al., 2014b; Neumann et al., 2015; Wattananit et al., 2016). Considering the growing importance of the immune response for stroke progression and recovery, it seems indispensable to scrutinize possible drug candidates for immunological (side) effects.

One compelling example is the peptide hormone granulocyte colony stimulating factor (GCSF) that recently missed study endpoints in a large multicenter clinical stroke trial (Ringelstein et al., 2013). This failure was to some extent unexpected as data regarding the efficacy in rodents belonged to the most convincing of any stroke drug candidate that had entered clinical trials (Minnerup et al., 2008). We recently hypothesized that dose-dependent immunological side effects of GCSF might have hampered successful translation into the clinic (Wagner et al., 2014). When back-calculating the clinically used dose to mice, GCSF significantly increased numbers of circulating regulatory T cells 24 h

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after stroke and thus may have amplified microvascular thrombosis in the ischemic penumbra (Kleinschnitz et al., 2013). Others demonstrated that GCSF potentiates the risk of hemorrhage in experimental stroke when used in combination with recombinant tissue plasminogen activator (Gautier et al., 2014). However, apart from the failure to meet study endpoints, AXIS 2 provided evidence for an inhibitory treatment effect on secondary infarct growth (Ringelstein et al., 2013). This finding, together with the promising preclinical records, tempted us to speculate about favorable immunomodulatory actions of GCSF in the delayed stage (>24 h) of stroke.

In fact, numerous studies have reported on immuno-regulatory and anti-inflammatory effects of GCSF, for instance in graft-versus-host disease or tumor models (D'Aveni et al., 2015; Joo et al., 2009; Kawano et al., 2015; Waight et al., 2013). High plasma levels of GCSF in response to infections or following external systemic administration markedly stimulate the differentiation and release of PMN and monocytes from the bone marrow. However, while fostering PMN effector functions, GCSF also seems to promote immunosuppressive and tolerogenic properties in monocytes and monocyte-derived cells (Boneberg et al., 2000; Carulli, 1997; Hartung et al., 1995; Martins et al., 2010; Mielcarek et al., 1998; Saito et al., 2002).

In mice, circulating monocytes can be classified into two functionally distinct subsets, namely inflammatory Ly6C+ monocytes that have a short half-life and are rapidly recruited to inflamed tissue (Geissmann et al., 2003) and the Ly6C− subset that provides vascular surveillance and orchestrates disposal of damaged endothelial cells (Auffray et al., 2007). After stroke, both monocyte subsets are present in the ischemic brain (Kim et al., 2014), but Ly6C− monocytes seem to play a negligible role for stroke development (Michaud et al., 2014). By contrast, the results of several studies indicate a harmful role of inflammatory monocytes, at least in the early stage of ischemic stroke (Dimitrijevic et al., 2007; Hammond et al., 2014b; Hughes et al., 2002; Schilling et al., 2009).

In this study, we thus investigated whether systemic administration of GCSF attenuates post-stroke inflammation by modulating monocyte responses. In fact, we found that GCSF strongly reduced monocyte/macrophage infiltration into the ischemic brain despite considerably increased blood monocyte counts. Lower numbers of mononuclear phagocytes in the brain translated to smaller brain edema and improved motor function. In vitro experiments further revealed that GCSF-induced interleukin (IL)-10 secretion suppresses integrin expression on activated mononuclear cells in an autocrine manner and may thus impair brain infiltration by promoting a less-adhesive monocyte phenotype.

2. Materials and methods

2.1. Mice

Investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were performed according to the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). All animal procedures were approved by the appropriate state agency (protocol numbers TVV12/11 and T65/13). A total of 78 male mice (C57BL/6JRj, Janvier Labs, Le Genest-Saint-Isle, France, 10–12 weeks old, body weight 25–30 g) were enrolled in the stroke study. Animals were randomly assigned to either sham surgery or middle cerebral artery occlusion (MCAO) with vehicle (5% glucose; hereafter termed "stroke") or GCSF treatment (hereafter termed "GCSF") and subsequently divided into experimental subgroups. Fig. 1 A illustrates the treatment regimen and timing of end point analyses. Naive mice were used to obtain control brain tissue for flow cytometry (n = 3) and to donate bone marrow cells for in vitro experiments (n = 6) (see below). Surgery, behavioral tests and evaluation of all readout parameters were performed by investigators blinded to the experimental groups.

2.2. Stroke model

Focal cerebral ischemia was elicited by transient MCAO as described previously (Kleinschnitz et al., 2006). Briefly, anesthesia was induced with 5% per volume (vol%) isoflurane and maintained with 2vol% isoflurane in oxygen-enriched air using a face mask. Body temperature was maintained at 36.5 °C ± 0.5 °C by a feedback-controlled heating device. Following a midline neck incision, the right common carotid and the right external carotid artery were ligated. Afterwards, a standardized silicon-rubber coated

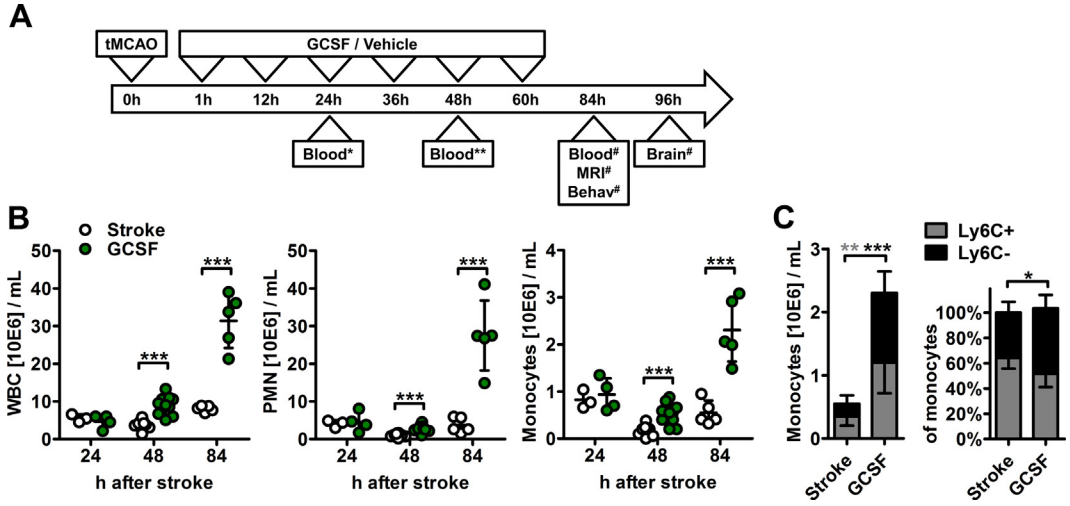

Fig. 1. Repeated systemic GCSF treatment induces granulocytosis and monocytosis in mice subjected to stroke. (A) Scheme illustrating the treatment regimen and timing of end point analyses. Samples were taken in three different experimental approaches (∗, ∗∗ and #). (B). Analysis of peripheral blood samples revealed a moderate increase of white blood cells (WBC) after 48 h and a strong increase at 84 h after stroke onset. This increase was primarily caused by polymorphonuclear neutrophils (PMN) and to a lesser extent by monocytes. (C) At 84 h after stroke, monocytes were further differentiated into Ly6C+ inflammatory monocytes and Ly6C− classical monocytes by flow cytometry. Treatment with GCSF significantly skewed the monocyte distribution towards the classical phenotype. Data are mean ± standard deviation for n = 3–10 (B) or n = 5 (C) animals per experimental group. $^{*}p < 0.05$, $^{***}p < 0.001$ by unpaired Student's *t* test.