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Suppression of dendritic cell functions contributes to the anti-inflammatory action of granulocyte-colony stimulating factor in experimental stroke

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ABSTRACT

Cerebral ischemia provokes an inflammatory cascade, which is assumed to secondarily worsen ischemic tissue damage. Linking adaptive and innate immunity dendritic cells (DCs) are key regulators of the immune system. The hematopoietic factor G-CSF is able to modulate DC-mediated immune processes. Although G-CSF is under investigation for the treatment of stroke, only limited information exists about its effects on stroke-induced inflammation. Therefore, we investigated the impact of G-CSF on cerebral DC migration and maturation as well as on the mediated immune response in an experimental stroke model in rats by means of transient middle cerebral artery occlusion (tMCAO). Immunohistochemistry and quantitative PCR were performed of the ischemic brain and flow cytometrical analysis of peripheral blood. G-CSF led to a reduction of the infarct size and an improved neurological outcome. Immunohistochemistry confirmed a reduced migration of DCs and mature antigen-presenting cells after G-CSF treatment. Compared to the untreated tMCAO group, G-CSF led to an inhibited DC activation and maturation. This was shown by a significantly decreased cerebral transcription of TLR2 and the DC maturation markers, CD83 and CD86, as well as by an inhibition of stroke-induced increase in immunocompetent DCs ($OX62^+OX6^+$) in peripheral blood. Cerebral expression of the proinflammatory cytokine TNF-α was reduced, indicating an attenuation of cerebral inflammation. Our data suggest an induction of DC migration and maturation under ischemic conditions and identify DCs as a potential target to modulate postischemic cerebral inflammation. Suppression of both enhanced DC migration and maturation might contribute to the neuroprotective action of G-CSF in experimental stroke.

Introduction

Acute focal cerebral ischemia leads to a complex interaction of inflammatory processes and immunoregulation (Iadecola and Alexander, 2001). Inflammatory cells invade the brain and mediate secondary brain injury (del Zoppo et al., 2001). At the same time, the so-called central immunodepression syndrome (CIDS) is induced. CIDS might represent a potential endogenous mechanism to limit harmful cerebral inflammation and overshot of immune response (Meisel et al., 2005; Prass et al., 2003). Clinically, CIDS is associated with post-stroke infectious complications which can worsen neurological outcome and increase mortality rates (Dirnagl et al., 2007; Harms et al., 2008). Therefore, therapeutic interventions which limit cerebral inflammation and CIDS, and are at the same time neuroprotective, should ensure clinical benfefits. Modulation of dendritic cell (DC) response might represent such a promising approach, since DCs are essential regulators of the immune system. Migrating into peripheral tissues, DCs scan the environment for invaded pathogens. Upon a contact with pathogenic structures, DC maturation is induced, characterized by an increased expression of co-stimulatory molecules (Banchereau and Steinman, 1998). Matured DCs are capable of activating T lymphocytes, which in turn are recruited to the inflamed tissue (Banchereau and Steinman, 1998). Former studies discovered the involvement of DCs in several neuroinflammatory disorders, including multiple sclerosis and Alzheimer's disease (Butovsky et al., 2007; Greter et al., 2005; Serafini et al., 2006).

Although the studies regarding DC's involvement in neuroinflammatory processes are just in their beginning, modulation of DC-functions has turned out to be a successful approach to counteract the harmful effects of neuroinflammation (Ganea et al., 2011; Ilarregui and Rabinovich, 2010; Manuel et al., 2007; Zinser et al., 2004). However, only limited information exists so far about the potential role of DCs

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in postischemic inflammation after focal cerebral ischemia (Kostulas et al., 2002; Reichmann et al., 2002; Yilmaz et al., 2010).

Granulocyte-colony stimulating factor (G-CSF) is known to alter DC response (Reddy et al., 2000; Talarn et al., 2006). As a neuroprotective growth factor, G-CSF has been successfully tested after experimental stroke (Schabitz et al., 2003; Sevimli et al., 2009). Though its anti-inflammatory effects have been shown to contribute to the neuroprotective action (Solaroglu et al., 2009), the detailed mechanisms of its interaction with the immune system in ischemic stroke are so far unknown. Investigating the immunomodulative capacity of G-CSF, our study focused on its effects on DCs and DC-mediated inflammation in experimental cerebral ischemia.

Materials and methods

Animals and experimental groups

All experiments were performed in accordance with European Communities Council Directive (86/609/EEC), and were approved by the local ethics committee. Male Wistar rats weighing 240–320 g (Charles River, Sulzfeld, Germany) were singly housed under controlled temperature with free access to food and water and kept on a 12-h light/12-h dark cycle.

Animals were randomly assigned to the following 3 groups: sham-surgery (n = 20); tMCAO (transient middle cerebral artery occlusion) (n = 15) with saline treatment (throughout the manuscript referred to as tMCAO group) and tMCAO with G-CSF application (n = 15) (Table 1). The applied dosage of G-CSF (60 μg/kg body wt) was chosen based on former studies (Schabitz et al., 2003). To assure randomization, treatment of each animal was allocated by a blinded investigator by lot.

To reduce stress and pain in the animals, anesthesia was induced by inhalation of a gas mixture of 4% isoflurane, oxygen (30%), and air, using a precalibrated vaporizer (Fortec; Cyprane Keighley, UK) and maintained by intramuscular injection of 100 mg/kg body weight ketamine hydrochloride and 6 mg/kg body weight xylazine. If necessary the anesthesia was sustained with further injections of 30 mg/kg body weight ketamine hydrochloride. Rectal temperature of the animals was monitored and maintained at a target body temperature of 37 °C by a heating pad, which included a feed-back mechanism for temperature control (Föhr Medical Instruments, Seeheim, Germany). Focal cerebral ischemia was induced by transient occlusion of the middle cerebral artery (tMCAO) for 60 min, using a 6–0 nylon filament as described elsewhere (Kollmar et al., 2002; Longa et al., 1989). Briefly, the right common carotid artery and the external carotid artery were exposed, a silicon-coated 6–0 monofilament nylon suture (Ethicon, Norderstedt, Germany) coated with silicon (Bayer, Leverkusen, Germany) was inserted into the distal right common carotid artery and carefully advanced into the internal carotid artery, approximately 18 mm from the carotid bifurcation. By use of this technique, the tip of the suture occludes unilaterally the proximal anterior cerebral artery, the origins of the MCA, and the posterior communicating artery. After 60 min of occlusion, reperfusion was induced by withdrawing the filament. Sham-surgery group underwent same experimental procedures as described above, except the intravascular filament. The injection of recombinant human G-CSF (Neupogen®, Amgen, Munich, Germany) or saline was applied once 30 min after induced tMCAO as described in Table 1.

In accordance to local ethics regulations, animals with a weight loss greater than 25% had to be killed to avoid further suffering during the remaining course of the study. In our study, two animals (1 saline-treated, 1 G-CSF-treated) had to be excluded from the experimental groups because of this reason.

Table 1
Treatment groups. Animals were randomly divided into one of the following groups: A) sham-surgery (n = 20), B) tMCAO (n = 15), C) tMCAO + G-CSF injection (n = 15). tMCAO = transient middle cerebral artery occlusion, G-CSF = granulocyte-colony stimulating factor, bw = body weight, i.p. = intraperitoneal.

Group	Surgery	Medical treatment	Route of application
A) Sham	No occlusion	0.5 ml NaCl	i.p., 30 min after sham-surgery
B) tMCAO	tMCAO (60 min)	0.5 ml NaCl	i.p., 30 min after beginning of occlusion
C) tMCAO+ G-CSF	tMCAO (60 min)	Single bolus of 60 μg/kg bw G-CSF	i.p., 30 min after beginning of occlusion

Neurological outcome

On the 7th day of post-tMCAO, the neurological outcome from all animals was assessed prior to euthanasia using a neuroscore (Menzies et al., 1992). According to this score, which has been shown to be correlated to the infarct size, the neurological deficit is classified into a rating scale from 0 to 4: 0 (no apparent deficit), 1 (contralateral forelimb flexion), 2 (decreased grip of contralateral forelimb while tail is pulled), 3 (spontaneous movement in all directions, contralateral circling only if tail is pulled), 4 (spontaneous contralateral circling). The testing was performed by a coworker, blinded to the treatment regimens.

Determination of infarct size

The animals were anesthetized and perfused with saline. Following euthanasia, brains were removed, snap-frozen, and kept at −80 °C until analyzed.

According to local ethics, animals with a weight loss greater than 25% were excluded from the study.

Silver infarct staining (SIS) (Vogel et al., 1999) was conducted on 20 μm thick coronal cryosections at six different brain levels 2 mm apart from each other (+5.2 mm to −1.2 mm from the bregma). Ischemic areas were measured in a blinded manner using a digital imaging analysis system (Analysis, Olympus).

The areas of the ischemic and nonischemic hemisphere were multiplied by the slice thickness to calculate the volume of the hemispheres. To determine the cerebral infarct volume, a modified version of the semi-automated method was used and calculated with an image analyzer (BioScan Optimas) by the following formula: CIV = NIH − (IH − IV), where CIV is corrected infarct volume; NIH, the volume of the nonischemic hemisphere; IH, volume of the ischemic hemisphere; and IV infarct volume.

Immunohistochemistry

To detect brain-migrated inflammatory cells, immunohistochemical staining was carried out on 10 μm thick acetone-fixed coronal cryosections. Two sections from each animal (+3.2 mm and +2.2 mm from the bregma, n = 8/group) were analyzed for each of the listed antibodies. Sections were stained with antibodies including anti-OX62 (DC marker, 1:50, Serotec, Düsseldorf, Germany), anti-CD86 (maturation marker, 1:400, Abcam, Cambridge, UK), anti-CD3 (T lymphocyte marker 1:500, DAKO, Hamburg, Germany) and anti-CD68 (macrophage/microglia marker, 1:50, Serotec). Since OX62 antibody also binds to a subpopulation of T lymphocytes, double-staining for OX62 and CD3 was performed on a serial section and double-positive cells were excluded from the analysis. CSA-Kit (DAKO) was used without the biotinylated tyramide amplification step. Briefly, endogenous peroxidase, biotin, avidin, and non-specific binding were blocked. Subsequently, the sections were incubated with the primary antibodies or the corresponding isotype Ig as negative control for 15 min, followed by 15 min incubation with biotinylated secondary antibody (1:5, DAKO) and 30 min horseradish peroxidase (HRP)-conjugated streptavidin–biotin-complex. The signal was visualized using diaminobenzidine (DAB) as a chromogen. Nuclei were counterstained