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Endogenous brain protection by granulocyte-colony stimulating factor after ischemic stroke

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ABSTRACT

Several lines of evidence have demonstrated beneficial effects of the hematopoietic factor G-CSF in experimental stroke. A conclusive demonstration of this effect in G-CSF deficient mice is, however, lacking. We therefore investigated the effect of G-CSF deficiency on infarct volumes, functional recovery, mRNA and protein expression of the matrix metalloproteinase 9 (MMP-9) after stroke. Furthermore we tested the efficacy of G-CSF substitution in G-CSF deficient animals to prevent the potential consequences of G-CSF deficiency. In the present study experimental stroke was induced in female non-treated wildtype (wt), G-CSF deficient mice and G-CSF substituted G-CSF deficient mice followed by assessment of infarct volumes, neurological outcome and sensorimotor function. In addition, immunohistochemistry and real-time PCR of the peri-ischemic area were performed. G-CSF deficient mice showed increased infarct volumes, whereas G-CSF substituted mice had a remarkable reduction in lesion size compared to wt mice. These findings are accompanied by an improvement in neurological and sensorimotor function. G-CSF deficiency resulted in an upregulation of MMP-9 in the direct peri-ischemic tissue. Treatment with G-CSF suppressed the upregulation of MMP-9. Taken together, G-CSF deficiency clearly resulted in enlarged infarct volumes, and worsened neurological outcome. G-CSF substitution abolished these negative effects, led to significant reduced lesion volumes, and improved neurological outcome. G-CSF mediated suppression of MMP-9 further demonstrates that endogenous G-CSF plays a significant role in brain protective mechanisms. We have shown for the first time that endogenous G-CSF is required for brain recovery mechanisms after stroke.

Introduction

Ischemic stroke caused by cerebral artery occlusion results in the activation of a complex cascade of pathophysiologic events, including brain edema, blood–brain barrier disruption, and neuroinflammation (Hallenbeck, 2002). These events crucially determine the extent of the final infarction (van Gijn and Dennis, 1998). Therapeutic interventions are remarkably limited and the number of patients afflicted with cerebral ischemia is steadily increasing. In spite of beneficial effects of thrombolysis, only a small percent of acute stroke patients qualifies for this specific therapy (Heuschmann et al., 2004).

The hematopoietic factor G-CSF was recently discovered to act as a protective and neurotrophic factor in the brain. Several groups described infarct reducing (Six et al., 2003; Schabitz et al., 2003; Gibson et al., 2005a) and recovery enhancing (Schneider et al., 2005) effects after ischemic stroke. G-CSF is a 19.6 kDa glycoprotein that regulates the generation, proliferation, survival, and maturation of neutrophilic granulocytes (Welte et al., 1985). It is in clinical use for more than 10 years for the treatment of neutropenia (Metcalf, 1990) as well as for bone-marrow reconstitution (Begley et al., 1986) and stem cell mobilisation (Weaver et al., 1993). The main actions of G-CSF are mediated via binding to the G-CSF receptor present on hematopoietic cells and as recently shown on neuronal cells (Schabitz et al., 2003). Due to its presence on neuronal cells and its upregulation after cerebral ischemia, an endogenous brain protective mechanism of action of this hematopoietic factor was hypothesized.

In the present study, we used a mouse model of G-CSF deficiency to address a number of key questions. First, we allocated adult female mice to evaluate the effect of G-CSF deficiency on lesion volume and post-stroke recovery. Furthermore, we investigated the role of the endogenous G-CSF system in post-ischemic recovery mechanisms by examining the effect of MMP-9 in the peri-ischemic mouse cortex, extracted by

Abbreviations: G-CSF, Granulocyte-colony stimulating factor; MMP-9, Matrix metalloproteinase 9; MCAO, Middle cerebral artery occlusion; LCM, Laser-capture microdissection; PCR, Polymerase chain reaction.

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laser-capture microdissection (LCM) and analysed by real-time polymerase chain reaction (PCR) and immunohistochemistry.

Materials and methods

Animal stroke model

All experiments were performed using an institutionally approved protocol following the governmental authorities. A total of 60 adult female mice (Jackson Laboratories) weighing 20–30 g were used in this study. The outbred G-CSF deficient ($n=40$) and wt ($n=20$) mice were derived from C57BL/6 and 129 inbred strains. G-CSF deficient mice were generated by targeted disruption of the G-CSF gene in embryonal stem cells (Lieschke et al., 1994). Mice were allowed free access to water and food before surgery. Anesthesia was induced with 2% and maintained with 1% halothane in a mixture of 70% nitrous oxide and 30% oxygen. A modified standard intraluminal filament technique was used to induce transient focal cerebral ischemia (Hata et al., 1998).

The left middle cerebral artery occlusion (MCAO) was induced by a 8-0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) and coated with silicon resin (Xantopren; Heraeus, Dormagen, Germany). Cerebral blood flow was continuously monitored using a laser Doppler probe (Periflux 5001; Perimed, Stockholm, Sweden) to verify ischemia and reperfusion. During the experiment rectal temperatures were maintained at 37 °C ± 0.5 °C with a thermostat-controlled heating pad. After 45 min, the filament was withdrawn to reperfuse the ischemic brain and the animals were allowed to recover from anesthesia.

G-CSF administration and experimental design

Animals were assigned in a randomized and blinded fashion to the following groups. Mice were allocated in three groups: non-treated wildtype, G-CSF deficient mice, and G-CSF deficient mice which received recombinant human G-CSF (NEUPOGEN; Amgen Inc.) by twice daily subcutaneous injections at a dose of 250 μg/kg (four days prior to surgery and continued for two days after ischemia) (Fig. 1). We did not include a G-CSF treated wt group in our study as the study is designed to identify any role for endogenous G-CSF by examining the effects of G-CSF deficiency. In addition, the efficacy of a G-CSF treatment has been extensively studied in wt animals.

Behavioral tests such as neurological score and rotarod performance were evaluated at 24 and 48 h after ischemia induction to evaluate the degree of hemiparesis and coordinated movements. 48 h after cerebral ischemia mice were deeply anesthesized and transcardially perfused with 50 ml of saline. Blood samples were obtained prior to perfusion using EDTA-coated microhematocrit tubes and total leukocyte count was performed on a automated counter. A total of 36 animals were used to carry out the above mentioned experiments ($n=12$ per group).

For detailed analysis of this hematopoietic factors brain protective capabilities, real-time PCR was performed to determine the effect of MMP-9 in peri-ischemic mouse cortex using a laser-capture microdissection ($n=8$ per group). Immunohistochemistry was performed on brain sections used for LCM-assisted tissue extraction to identify MMP-9 positive signals.

Determination of infarct volume

Mice were sacrificed for measurement of brain swelling and infarct volumes 48 h after induction of MCA occlusion, shortly after the last neurologic evaluation. Brains were removed and frozen in powdered dry ice. To quantify ischemic damage, coronal brain sections (thickness, 10 μm) were serially cut in a cryostat, collected at 100 μm intervals, and stained with toluidine blue (Sigma, St. Louis, USA). Infarct volume was quantified with a standard computer assisted image analysis technique. To compensate for the effect of brain edema, the corrected infarct volume was calculated. The correction method is based on the determination of ischemic swelling by comparing the volume of ischemic and non-ischemic hemispheres. In addition brain swelling was analysed by measuring the percentage of hemispheric enlargement ((ischemic hemisphere area) − (contralateral hemisphere area) / (contralateral hemisphere area) * 100).

Neurological evaluation

A neurological test was performed by an observer (JKS) who was blinded to the experimental groups at 24 and 48 h after MCAO. According to the test, neurological deficits were scored on a 0–5 scale: normal motor function (0); decreased grip of contralateral forelimb grip while tail pulled (1); flexion of torso and contralateral forelimb when mouse was lifted by the tail (2); circling to the contralateral side when mouse was held by the tail on a flat surface, but normal posture at rest (3); no spontaneous motor activity (4); dead (5).

Rotarod test

To characterize an improvement in sensorimotor activity of mice after MCAO a rotarod with a 30 mm diameter rod and acceleration capability was used. Mice were acclimatized to the rotarod 2 days before surgery. For each session mice were subjected to 5 rotarod trials. Each mouse was placed on a drum with an accelerating speed of 4–40 rpm over a 3-min period. The trial ended and was recorded when the experimental mice fell off the drum. Each mouse underwent testing prior surgery for baseline performance, 24 and 48 h after MCAO.

Laser microdissection and real-time PCR

Frozen brain blocks were taken to a cryostat (Leica, Nussloch, Germany) and 10 μm coronal sections were obtained and placed on PEN-membrane 1 mm glass slides (P.A.L.M. Bernried, Germany). Laser microdissection of peri-ischemic cortex (Fig. 1) was performed using an AS LMD System equipped with a UV laser (Leica Microsystems GmbH, Wetzlar, Germany), using a 10× magnification. For each sample, cortex tissue from 90 consecutive coronal cryosections was collected and combined in one tube cap, which was filled with lysis buffer (Buffer RLT, RNeasy Micro Kit, Quiagen, Hildesheim, Germany). Total RNA from these samples was isolated using the RNeasy Micro Kit (Quiagen) according to manufacturer instructions. All steps were carried out under RNase-free conditions. RNA quality and quantity were checked by analysing 1 μl of RNA on the Agilent 2100 Bioanalyzer using the RNA 6000 Pico LabChip Kit (Agilent Technologies).

Real-time PCR was performed on laser captured tissue of the peri-ischemic cortex to analyse levels of MMP-9 mRNA in wt, G-CSF deficient and G-CSF treated mice at 48 h after cerebral ischemia. The LightCycler PCR analysis was performed using the SYBR Green master mix, according to the manufacturer recommendations (Roche Diagnostics Corp.). Specificity of product was ensured by melting point analysis and agarose gel electrophoresis. The following primer pairs were used: MMP9-2855s, CAAGGATTGCTCAGAGATTCTCCG; MMP9-3049as, ATCTCACCTGGAGGACACAGTCTG. cDNA content of samples was normalized to the expression level of the housekeeping gene *cyclophilin* (primers: cyc5, ACCCCACCGTGTTCTTCGAC; acyc300, CATTTGCCATGGACAAGATG). Relative regulation levels were derived after normalization to *cyclophilin*.

Immunohistochemistry and image analysis

Immunohistochemistry was performed in MCAO mice on 10 μm-thick frozen coronal sections using MMP-9 rabbit polyclonal antibody (1:100, Abcam, Cambridge, UK) and an appropriate secondary