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MCP-1/CCR-2-double-deficiency severely impairs the migration of hematogenous inflammatory cells following transient cerebral ischemia in mice

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ABSTRACT

Monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR-2 are known to play a major role in inflammatory responses after cerebral ischemia. Mice deficient in either MCP-1 or CCR-2 have been reported to develop smaller infarct sizes and show decreased numbers of infiltrating inflammatory cells. In the present study we used green fluorescent protein (GFP) transgenic mice to investigate the effect of MCP-1/CCR-2-double deficiency on the recruitment of inflammatory cells in a model of both, mild and severe cerebral ischemia. We show that MCP-1/CCR-2-double deficiency virtually entirely abrogates the recruitment of hematogenous macrophages and significantly reduces neutrophil migration to the ischemic brain 4 and 7 days following focal cerebral ischemia. This argues for a predominant role of the MCP-1/CCR-2 axis in chemotaxis of monocytes despite a wide redundancy in the chemokine-receptor-system. Chemokine analysis revealed that even candidates known to be involved in monocyte and neutrophil recruitment like MIP-1α, CXCL-1, C5a, G-CSF and GM-CSF showed a reduced and delayed or even a lack of relevant compensatory response in MCP-$1^{-/-}$/CCR-$2^{-/-}$-mice. Solely, chemokine receptor 5 (CCR-5) increased early in both, but rose above wildtype levels at day 7 in MCP-$1^{-/-}$/CCR-$2^{-/-}$-animals, which might explain the higher number of activated microglial cells compared to control mice. Our study was, however, not powered to investigate infarct volumes. Further studies are needed to clarify whether these mechanisms of inflammatory cell recruitment might be essential for early infarct development and final infarct size and to evaluate potential therapeutic implications.

Introduction

The inflammatory response following ischemic brain injury is assumed to contribute significantly to the pathogenesis and outcome of stroke. This phase is characterized by activation of local immune cells, secretion of inflammatory molecules and recruitment of different types of inflammatory cells to the CNS and is supposed to be more amenable to therapeutic options than acute neurotoxicity (Jin et al., 2010; Moxon-Emre and Schlichter, 2010). Therefore the temporal pattern of immune cell activation and infiltration following brain ischemia has been of recent interest and intensively studied. However, the understanding of postischemic inflammatory mechanisms is still limited and the time-dependent recruitment of inflammatory cells controversially discussed (Jin et al., 2010). Monocyte chemoattractant protein-1 (MCP-1) is a member of the CC-chemokine subfamily and has been shown to play an important role in the recruitment of inflammatory cells to the CNS (Babcock et al., 2003; Jiang et al., 2008). Increased MCP-1 levels have been detected after ischemic brain injury and suggested to contribute to the ischemic damage by promoting immigration of inflammatory cells (Che et al., 2001; Chen et al., 2003). MCP-1 exerts its activity via exclusive and high-affinity binding to the CCR-2 receptor. In an experimental stroke model CCR-2-deficiency leads to reduced infarct size and diminished infiltration of inflammatory cells (Dimitrijevic et al., 2007). Using a model of green fluorescent protein (GFP) transgenic bone marrow chimeric mice to distinguish resident microglia cells from hematogenous macrophages we previously reported that both MCP-1- and CCR-2-deficient mice show decreased recruitment of hematogenous macrophages to the ischemic brain (Schilling et al., 2009b,c) underlining the crucial role of this pathway in monocyte migration. Based on our recent findings, showing a reduced turnover rate of perivascular and meningeal macrophages in MCP-1- and CCR-2-double-deficient mice (Schilling et al., 2009a), we investigated in the present study the role of combined MCP-1/CCR-2-deficiency in recruitment of inflammatory cells after transient focal ischemia.

Materials and methods

Animals

All animal studies and procedures have been approved by the local governmental authorities (Landesamt für Natur, Umwelt und Verbraucherschutz, NRW, Germany) and were performed in accordance with the European convention for animal care and ethical use of

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laboratory animals. MCP-1-deficient mice were obtained from The Jackson Laboratory (Bar Harbor, USA). Disruption of the MCP-1 gene locus has been previously described by Lu et al. (1998). CCR-2-deficient mice were generously donated by W.A. Kuziel (Kuziel et al., 1997). GFP-transgenic mice (C57BL/6-GFP) were characterized by Okabe et al. (1997) and wildtype male C57BL/6-mice (MCP-$1^{+/+}$/CCR-$2^{+/+}$) were purchased from Charles-River (Sulzfeld, Germany). Knock-out-mice were backcrossed at least eight times into a C57BL/6 background. MCP-1/CCR-2 double-knockout mice (MCP-$1^{-/-}$/CCR-$2^{-/-}$) were generated by crossing MCP-1-deficient and CCR-2-deficient mice for at least five times. Genotyping was done, according to Jax® Mice genotyping protocols, via PCR (polymerase chain reaction) using DNA extracted from tail samples.

Production of bone marrow chimeric mice

Homozygous MCP-$1^{-/-}$/CCR-$2^{-/-}$- and MCP-$1^{+/+}$/CCR-$2^{+/+}$-mice were crossed with GFP-transgenic mice (MCP-$1^{+/+}$/CCR-$2^{+/+}$-GFP$^{+/+}$) to generate suitable bone-marrow donors. Only male bone marrow chimeric mice were generated for subsequent experiments. MCP-$1^{-/-}$/CCR-$2^{-/-}$-GFP$^{+/+}$-animals and MCP-$1^{+/+}$/CCR-$2^{+/+}$-GFP$^{+/+}$-littermate donors were sacrificed under deep ketamine/xylazine anesthesia by cervical dislocation. Bone marrow was obtained by flushing the femur veins with sterile phosphate buffered saline. Bone marrow cells were suspended in the same buffer, washed three times, counted and re-suspended at 1.2×10^8 cells/ml. Six to eight-week old male MCP-$1^{-/-}$/CCR-$2^{-/-}$-mice and MCP-$1^{+/+}$/CCR-$2^{+/+}$-controls (sub-lethally irradiated with 7 Gy in a cobalt source) served as bone marrow recipients. A head protection was not used during irradiation. Each irradiated animal received 100 µl of the cell suspension by injection into the tail vein. MCP-$1^{-/-}$/CCR-$2^{-/-}$-GFP$^{+/+}$-bone marrow was transplanted into the appropriate MCP-$1^{-/-}$/CCR-$2^{-/-}$-recipients. MCP-$1^{+/+}$/CCR-$2^{+/+}$-mice received MCP-$1^{+/+}$/CCR-$2^{+/+}$-GFP$^{+/+}$-bone marrow. Three months after recovery chimerism was controlled by counting numbers of GFP-positive and GFP-negative leukocytes in blood smears taken from each animal. Only animals with more than 90% GFP-positive leukocytes were used for further analyses.

Transient focal ischemia

Transient focal ischemia was induced by occlusion of the left middle cerebral artery (MCAO). Twenty chimeric MCP-$1^{-/-}$/CCR-$2^{-/-}$-GFP$^{+/+}$-mice and 20 chimeric MCP-$1^{+/+}$/CCR-$2^{+/+}$-GFP$^{+/+}$-controls underwent MCAO for 30 min. Survival times were 1, 2, 4 and 7 days ($n=5$ per group). Five sham-operated chimeric MCP-$1^{-/-}$/CCR-$2^{-/-}$-GFP$^{+/+}$-animals and five chimeric MCP-$1^{+/+}$/CCR-$2^{+/+}$-GFP$^{+/+}$-controls served as baseline (day 0). To determine the effect of more severe ischemia, five chimeric knock-out-mice and five corresponding chimeric controls were subjected to MCAO for 60 min. As evolution of post-ischemic damage is completed 1 day after MCAO (Hata et al., 2000) and to prevent an increase in postoperative mortality, a survival time of 1 day was chosen for animals which underwent 60 min of cerebral artery occlusion. MCAO was performed using a modified intraluminal filament technique (Hata et al., 1998) under 1.5% isoflurane anesthesia in 30% O_2/70% N_2O. Rectal temperature was measured throughout surgery and maintained at 37 °C ± 0.5 °C. In brief, left common carotid artery was exposed through a midline incision in the neck and incised. Then an 8-0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with a silicon resin (Xantopren; Heraeus, Dormagen, Germany) was inserted and advanced 9 mm distal to the carotid bifurcation. Temporary occlusion of the MCA and following reperfusion was detected by a laser Doppler probe (Periflux 5001; Perimed, Stockholm, Sweden) for continuous measurement of cerebral blood flow (CBF). A CBF drop below 20% of pre-occlusion flow confirmed correct placement of the filament, followed by adequate reperfusion after thread withdrawal. Body temperature of the mice was continuously measured.

Tissue preparation

Animals were perfused, under deep ketamine/xylazine anesthesia, through the left ventricle for 1 min with a 6% hydroxyethyl–starch solution (HAES steril; Fresenius, Bad Homburg, Germany) followed by 4% buffered paraformaldehyde (PFA, pH 7.4, 10 min). Brains were rapidly removed, postfixed in 4% buffered PFA for 3 hours, immersed in 10% sucrose overnight, frozen and stored at −80 °C until further use.

Nissl staining

To compare the brain structure between MCP-$1^{+}/^{+}$/CCR-$2^{+}/^{+}$ and MCP-$1^{-}/^{-}$/CCR-$2^{-}/^{-}$-mice, we performed Nissl staining using cresyl violet. In brief, brain slices were air-dried for 60 min and incubated in 70% ethanol for 10 min. Staining was performed with a 0.1% cresyl violet (Sigma-Aldrich)/100% ethanol solution for 10 min. Dehydration was done through graded alcohols and cleared in Histoclear II® (Linaris, Germany). Finally, brain slices were coverslipped using permanent mounting medium Roti-Histokit II (Roth, Germany). The stained specimens of MCP-$1^{+}/^{+}$/CCR-$2^{+}/^{+}$ and MCP-$1^{-}/^{-}$/CCR-$2^{-}/^{-}$-animals were photographed using a Nikon Eclipse 80i microscope and were subsequently examined with regard to differences in overall brain structure.

Immunohistochemistry

Coronal sections within the infarcted area (approximately bregma 0.64 mm) were used for immunohistochemistry. Sections were pretreated with 30% H_2O_2/methanol (10 min) and subsequently incubated with Blocking Reagent (Roche Diagnostics, Mannheim, Germany) for 15 min to block endogenous peroxidase and nonspecific protein binding respectively. For detection of microglia and macrophages monoclonal antibody anti-F4/80 (diluted 1:500, Serotec, Biozol Diagnostica, Eching, Germany), directed against a 160 kDa transmembrane protein expressed by murine microglia and macrophages, was applied. Neutrophil granulocytes were detected using monoclonal antibody anti-7/4 (Serotec, Duesseldorf, Germany) diluted 1:200 directed against 7/4, a polymorphic 40 kDa protein expressed by polymorphonuclear cells. CCR-5 was investigated using rabbit-anti-CCR-5 antibody (1:100, Abcam, Cambridge, UK). Sections were incubated at 4 °C overnight. A biotinylated rabbit anti-rat antibody (Vector, Burlingame, CA, USA) applied for 45 min served as secondary antibody (diluted 1:100 for 7/4 and 1:200 for F4/80-antibody detection). Detection of anti-CCR-5 antibodies was done with a biotinylated goat anti-rabbit antibody (Jackson Labs, West Grove, PA) applied at a dilution of 1:200 for 45 min (room temperature). For signal amplification sections were treated with horseradish-peroxidase/streptavidin (DAKO, Glostrup, Denmark) diluted 1:100 for 45 min and biotinylated tyramide, diluted 1:100 for 10 min at room temperature. Visualization of antibodies was carried out using a streptavidin-conjugated fluorescence dye (Alexa Fluor™ 594, Molecular Probes, Leiden, the Netherlands, 1:100, 45 min at room temperature). Nuclear counterstain was done with a fluorescence-preserving mounting medium containing 4′6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA, USA).

Image analysis

Resident microglia, hematogenous macrophages and neutrophil granulocytes within the infarcted area were counted in six non-overlapping fields of 0.1 mm^2 covering almost the entire lateral caudate putamen. Although MCAO for 60 min caused a more widespread