Predominant phagocytic activity of resident microglia over hematogenous macrophages following transient focal cerebral ischemia: An investigation using green fluorescent protein transgenic bone marrow chimeric mice

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Abstract

Activated microglia and hematogenous macrophages are known to be involved in infarct development after cerebral ischemia. Traditionally, hematogenous macrophages are thought to be the primary cells to remove the ischemic cell debris. However, phagocytosis is a well-known property also of activated microglia. Due to a lack of discriminating cellular markers, the cellular origin of phagocytes and the temporal course of phagocytosis by these two cell types are largely unknown. In this study, we used green fluorescent protein (GFP) transgenic bone marrow chimeric mice and semithin serial sections after methyl methacrylate embedding of the brains to dissect in detail the proportion of identified activated resident microglial cells and infiltrating hematogenous macrophages in phagocytosing neuronal cell debris after 30 min of transient focal cerebral ischemia. Already at day one after reperfusion, we found a rapid decrease of neurons in the ischemic tissue reaching minimum numbers at day seven. Resident GFP-negative microglial cells rapidly became activated at day one and started to phagocytose neuronal material. By contrast, hematogenous macrophages incorporating neuronal cell debris were observed in the ischemic area not earlier than on day four. Quantitative analysis showed maximum numbers of phagocytes of local origin within 2 days and of blood-borne macrophages on day four. The majority of phagocytes in the infarct area were derived from local microglia, preceding and predominating over phagocytes of hematogenous origin. This recruitment reveals a remarkable predominance of local defense mechanisms for tissue clearance over immune cells arriving from the blood after ischemic damage.

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Introduction

After cerebral ischemia, complex pathophysiological events are involved in infarct development over time and space. Excitotoxicity, perifocal depolarization, inflammation and programmed cell death are the predominant mechanisms regulating neuronal survival and development of tissue damage and final infarct size after vessel occlusion (Dirnagl et al., 1999). The inflammatory response after focal cerebral ischemia is characterized by an extremely rapid activation of microglia/macrophages within hours (Morioka et al., 1993; Kato et al., 1996; Lehrmann et al., 1997; Zhang et al., 1994). Resident microglial cells and infiltrating hematogenous macrophages play an important role during the pathogenetic cascade following cerebral ischemia since they express a plethora of growth factors, chemokines and regulatory cytokines as well as free radicals and other toxic mediators (Raivich et al., 1999) which are involved in secondary infarct expansion (Del Zoppo et al., 2000; Hallenbeck, 2002). Further, microglial cells are essential as scavenger cells in tissue repair and are of functional importance since insufficient removal of cell debris has been identified as one of the major causes for regeneration failure (Stoll et al., 2004). Phagocytosis is stimulated by specific...
epitopes on phagocytic targets and requires activation of downstream signaling cascades that lead to the rearrangement of the actin cytoskeleton and incorporation of the cell debris (Koenigsknecht and Landreth, 2004). A large number of actin-regulatory proteins are responsible for the formation of multiform actin assemblies and macrophages have been reported to contain various actin-binding proteins (Ohsawa et al., 2004). However, little is known about factors that regulate microglial phagocytosis (Mitrasinovic et al., 2003). Traditionally, hematogenic macrophages are considered to be responsible for phagocytosis (Perry et al., 1987). But once activated, resident microglia and hematogenous macrophages are not distinguishable by morphological criteria due to a lack of discriminating cellular markers (Kreutzberg, 1996). Using GFP transgenic bone marrow chimeric mice, we were enabled to conduct a definitive distinction between these two cell types. Recently, we found a rapid activation of resident microglial cells and a remarkable delay of infiltration and unexpected small number of hematogenous macrophages after transient focal cerebral ischemia (Schilling et al., 2003). These findings evoke the hypothesis that microglial cells and infiltrating macrophages act differently in phagocytosis after ischemic stroke. In order to answer this question and to quantify the proportion of phagocytosis performed by hematogenous macrophages or activated microglial cells, we examined GFP transgenic bone marrow chimeric mice after transient focal cerebral ischemia using the methyl methacrylate embedding technique for immunohistochemical analysis of multiple antigens in semithin serial sections (Mueller et al., 2000).

Materials and methods

Production of bone marrow chimeric mice

The animal experiments were approved by the local governmental authorities. Male C57BL/6J-mice (20–30 g) were obtained from Charles-River (Sulzfeld, Germany). GFP-transgenic mice (C57BL/6J-GFP) were generously donated by Dr. Masaru Okabe, Osaka, Japan (Okabe et al., 1997). Bone marrow chimeric mice were created as described previously (Mueller et al., 2001). In brief, 6–8 weeks old male C57BL/6J-recipients (20–30 g) were sublethally irradiated with 8 Gy in a cobalt source. Male donor animals were killed under deep ether anesthesia by cervical dislocation. Bone marrow was obtained by flushing the femur bones with sterile phosphate buffered saline. Bone marrow cells were suspended in the same buffer, washed several times, counted and resuspended at 4 x 10⁷ cells/ml. The suspension (300 µl) was transplanted into each irradiated recipient animal through injection into the tail vein. After 3 months of recovery, all further experiments were done. Chimerism was controlled by counting the number of GFP-positive and GFP-negative leukocytes in blood smears taken from each animal. Only animals with greater than 90% GFP-positive leukocytes were used for further experiments.

Transient focal cerebral ischemia

Transient focal cerebral ischemia was induced by occlusion of the left middle cerebral artery (MCAO) applying a modified intraluminal filament technique (Hata et al., 1998) under inhalation anesthesia. To avoid large infarcts of the entire MCA-territory and a consecutive increase of postoperative mortality, a transient occlusion of 30 min was chosen. In brief, an 8–0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with silicon resin (Xantopren; Heraeus, Dormagen, Germany) was introduced through a small incision into the left common carotid artery and advanced approximately 9 mm distal to the carotid bifurcation for temporary occlusion of the MCA. Cerebral blood flow was continuously monitored using a laser Doppler probe (Periflux 5001; Perimed, Stockholm, Sweden) to verify ischemia and reperfusion. Sham operation was performed by insertion of the filament into the internal carotid artery with its tip proximal to the carotid canal.

Tissue preparation

Eighteen bone marrow chimeric mice (n = 3 per group) underwent transient focal cerebral ischemia. Survival times were 1, 2, 4, 7, 10 and 14 days. Three chimeric mice 3 months after bone marrow transplantation (day 0) and sham-operated chimeric mice (n = 2 per group at 2, 4 and 7 days) served as controls. The animals were perfused through the left ventricle for 1 min with a 6% hydroxyethyl-starch solution (HES steril; Fresenius, Bad Homburg, Germany) followed by 4% buffered paraformaldehyde (PFA) at pH 7.4 for 10 min under deep ether anesthesia. Brains were rapidly removed and postfixed in 4% buffered PFA for a further 3 h.

Methyl methacrylate embedding

Brain tissue from ischemic and sham-operated mice as well as control mice from day 0 was embedded in methyl methacrylate as described previously (Mueller et al., 2000). Briefly, tissue was dehydrated in pure acetone for 24 h at -20°C. After dehydration, the tissue was placed in solution MMA1 consisting of 6 ml of MMA (Sigma, Deisenhofen, Germany), 3.5 ml of butyl-methacrylate (Sigma, Deisenhofen, Germany), 500 µl of methyl-benzoate (Merck, Darmstadt, Germany) and 120 µl of polyethylene glycol 400 (Sigma, Deisenhofen, Germany) for 8 h. The tissue was then incubated for 8 h in solution MMA2 which is MMA1 containing an additional amount of 800 mg/100 ml dry benzoylperoxide (Sigma, Deisenhofen, Germany). Polymerization was allowed for 48 h at -20°C under vacuum in
solution MMA3 containing the mentioned substances above plus 600 µl/100 ml N,N-dimethyl-p-toluidine (Merck, Darmstadt, Germany). The blocks were cut on a Reichert-Jung ultracut ultramicrotome. Series of semithin sections were transferred onto coated glass slides and dried at 35°C for 2 h.

**Immunohistochemistry**

Coronal sections were deplasticized by incubation in pure acetone for 3 x 20 min, pretreated with citrate buffer in a microwave oven for 15 min and nonspecific protein bindings were blocked by incubation for 15 min in Blocking Reagent (Roche Diagnostics, Mannheim, Germany). Afterwards, sections were incubated with the following primary antibodies: NeuN, diluted 1:50 (Chemicon International, Temecula, CA, USA), recognizing vertebrate neuron-specific nuclear protein, or Iba-1, diluted 1:200 (generously donated by Dr. Yoshinori Imai, Tokyo, Japan), directed against murine microglia and macrophages at 4°C overnight. Secondary antibodies were applied for 45 min at room temperature. To detect anti-NeuN antibody, a biotinylated goat anti-mouse antibody was used at 1:100 (Dianova, Hamburg, Germany). To detect Iba-1-antibody, we used a biotinylated goat anti-rabbit antibody, applied at a dilution of 1:100 (Vector, Burlingame, CA, USA). For amplification of Iba-1 signals, the sections were pretreated with 30% H2O2 for 10 min to block endogenous peroxidases, and subsequently incubated 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. Antibodies were visualized by a conjugate of streptavidin and a fluorescent dye (Alexa Fluor® 594, Molecular Probes, Leiden, The Netherlands), diluted 1:100. Nuclear counterstaining was done using a fluorescence-preserving mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA, USA).

**Image analysis**

The sections were examined using a fluorescence microscope (Leica DM microscope, Bensheim, Germany) with appropriate filter sets for Alexa Fluor 594, DAPI or GFP. Sections were digitized with a Diagnostic Instruments SPOT II Advanced Camera System (Visitron, München, Germany). Analysis of microglia/macrophages and neuronal material incorporating phagocytes was possible by colocalization of immunofluorescent signals from NeuN- and Iba-1-antibodies or GFP in adjacent sections. In each section, neurons, microglial cells and hematogenous macrophages were counted in the infarct area in 5 random nonoverlapping fields covering almost the entire lateral caudate putamen. Data are presented as mean values ± SD (n = 3 animals per group except n = 6 for sham-operated animals). Student’s t test was used for statistical analyses.

**Results**

The evolution of ischemic damage after transient MCAO for 30 min reproducibly followed a profile of unilateral infarction within the lateral caudate putamen with little variation between different animals. Cerebral blood flow measurements confirmed a drop of cerebral blood flow below 15% after placement of the intraarterial thread, followed by adequate reperfusion following thread withdrawal. As mentioned above, only chimeric mice with nearly complete chimerism were used containing a minimum of 90% GFP-positive leukocytes in their blood smears. Consistent with previous results (Schilling et al., 2003), white blood counts were similar in chimeric animals and nonchimeric controls. Furthermore, neither the development of ischemic changes and final infarct size nor the evolution of the microglia/macrophage and astrocytic responses or granulocyte infiltration (data not shown) was different between chimeric animals or nonchimeric controls.

**Neuronal damage**

In the uninjured brain on day 0 as well as in sham-operated animals, we found normal numbers of neurons in the cortex (Fig. 1A) and the caudate putamen of the ipsilateral hemisphere (756 neurons/mm² on day 0). Already 1 day after MCAO, an obvious reduction of cells in the ischemic area could be seen, evoked by a marked decrease of neurons to less than one half compared to day 0. Assessing neuronal damage morphologically and further by the loss of NeuN-immunostaining, already on day one, many NeuN-positive cells had a shrunken cytoplasm and an irregular nucleus (Fig. 1B). In the following days, the number of neurons in the ischemic caudate putamen decreased continuously (Fig. 2). At day seven, we found only 6% neurons compared to day 0. At 2 weeks after MCAO, only solitary surviving neurons could be detected in the infarct area. In the boundary zone of the infarct and the adherent neocortex, the neurons had a normal morphology.

**Microglial activation**

In the striatum of animals without cerebral ischemia and in sham-operated mice, we did not find any changes in number (65 microglial cells/mm² on day 0) and morphology of microglial cells (Fig. 3A). There were no signs of microglial activation. At day one after MCAO, the earliest postischemic time point investigated, all Iba-1-positive cells were GFP-negative, indicating that no hematogenous macrophages entered the ischemic area. The number of microglial cells did not change significantly at day one, but they lost their thin ramification towards retracted processes and developed a more ameboid and rounded cell body (Fig. 3B). These morphological changes were intensified at day two and strongly activated Iba-1-positive cells persisted until 2 weeks after cerebral ischemia. Quantitative studies
(Fig. 2) revealed an increased number of microglial cells (Iba-1-positive and GFP-negative) at day 2 with a nearly 5-fold increase at day 7 compared to the uninjured brain. At day 10, we found a maximum of microglial cells in the striatum with a 7.5-fold increase compared to the number of microglia in the normal brain (day 0).

**Macrophage infiltration**

GFP-positive/Iba-1-positive hematogenous macrophages first entered the lateral caudate putamen at day two after reperfusion. These cells were morphologically indistinguishable from activated ameboid microglia but could be easily identified by the green fluorescence of GFP (Figs. 3B–E). The number of hematogenous macrophages increased to a maximum at day seven (75 cells/mm²) and decreased until day 14. At day seven, 20% of the total population of all Iba-1-positive cells in the ischemic area were hematogenous macrophages. But the majority of Iba-1-positive cells remained GFP-negative indicating their derivation from microglia (Fig. 2).

**Phagocytosis of neuronal debris by resident and infiltrating macrophages**

To analyze the phagocytic capacity of blood-borne monocyte derived macrophages and activated local microglia, we studied the incorporation of neuronal (NeuN-positive) material into identified activated microglia (Iba-1-positive/GFP-negative) and hematogenous macrophages (Iba-1-positive/GFP-positive) by co-localizing specific signals on adjacent semithin sections and quantifying identified phagocytic cells.

In animals without cerebral ischemia (day 0) and in sham-operated mice, phagocytosis of neuronal debris was not detectable. Already at day one after reperfusion, we found the first Iba-1-positive cells which had incorporated neuronal material (Figs. 5A–C). At day 2, the number of...
Phagocytic cells was similar to day one (20 cells/mm²). Quantitative analysis (Fig. 4) showed that the maximum of phagocytosis was already reached at these early time points. The complete absence of the GFP signal in Iba-1-positive phagocytic cells at these time points indicated that phagocytosis was exclusively performed by phagocytes derived from resident microglial cells. At day four, we observed a decrease of microglial phagocytes to nearly one half. At this day, we found for the first time Iba-1-positive, GFP-positive cells which had incorporated NeuN-positive material indicating that these phagocytes were of hematogenous origin (Figs. 5D–F).

The total number of phagocytic microglial cells stayed nearly at the same level from day four until day 10. Hematogenous macrophages infiltrated into the ischemic area had their maximum at day four, decreased at day seven and 10 and were no longer found 2 weeks after reperfusion. At day four, 15% of all phagocytes were of hematogenous origin. At all time points, the number of phagocytes within the total population of resident microglia or hematogenous macrophages was very small. At day one and day two, where we found peak numbers of phagocytosing microglia, these cells amount to only one quarter of the total number of microglial cells at these time points.

**Discussion**

Traditionally, hematogenic macrophages are thought to be the primary cell type for phagocytosing of cellular debris...
after cerebral ischemia and to promote scar formation (Mabuchi et al., 2000; Ito et al., 2001). In this study, we were able to dissect in detail the proportion of identified activated resident microglial cells phagocytosing neuronal cell debris after transient focal cerebral ischemia on the one hand and infiltrating hematogenous macrophages on the other. Until now, no quantitative data were available and no distinction was made between resident microglia and infiltrating hematogenous macrophages during phagocytosis following cerebral ischemia.

In parallel with the rapid activation of resident microglia, we found peak phagocytic activity in the first 2 days after reperfusion. There was no participation of hematogenous macrophages at these early time points. Peak phagocytic activity of infiltrating macrophages incorporating NeuN-positive material was identified at day four after cerebral ischemia but the absolute number of hematogenous phagocytes was surprisingly low.

According to previous experiments (Schilling et al., 2003) and consistent with several other investigators (Kato et al., 1996; Lehrmann et al., 1997; Zhang et al., 1994), we found an extremely rapid activation of local microglial cells within the infarct zone which was evident already at day one after disease onset, the earliest time point investigated in our experiments. Schroeter et al. (1997) summarized in a macrophage-depletion model that the initial macrophage response after photochemically induced ischemia in the perifocal region was of microglial origin while hematogenous macrophages were recruited with remarkable delay. Furthermore, Kleinschnitz et al. (2003) suggested from macropathology experiments by magnetic resonance imaging that major macrophage infiltration is delayed and occurs at a stage in which neuronal cell death is mostly completed. It was speculated that infiltrating macrophages play a role in tissue remodeling rather than in neuronal injury. This assumption matches our observation that the majority of neuronal damage, conspicuous by a continuous decrease of neurons in the ischemic striatum, occurs before hematogenous macrophages infiltrate the infarcted brain, and that hematogenous macrophages participate in phagocytosis only in a small amount compared to resident activated microglia.

Interestingly, the kinetics of microglial/macrophage numbers and the phagocytic activity of both types of macrophages are only partially concordant. Activated microglia increased during the first 10 days after cerebral ischemia while the maximum number of phagocytes from local origin was reached already within 2 days after vessel occlusion. Similarly, peak phagocytic activity by hematogenous macrophages precedes the time point of their highest numbers by 3 days (day four vs. day seven). Tanaka et al. (2003) also found hematogenous macrophages, morphologically characterized as phagocytes, infiltrating predominantly the ischemic core area at day seven in a similar GFP-bone marrow chimeric mouse model of permanent cerebral ischemia. Surprisingly, the number of activated microglial cells in the ischemic core or transition area was very low. In this study, phagocytosis was not investigated and no information was given about a distinction between phagocytes of microglial or hematogenous origin. Based on the low numbers of activated microglia in the transition area and the ischemic core in this study of permanent cerebral ischemia, it might be speculated that the duration of ischemia might have a major impact on the kinetics of...
phagocytic activity in transient cerebral ischemia of longer duration than under our own experimental conditions and may result in a reduction of phagocytosis by resident microglia and hematogenous macrophages.

In our experiments, we focused on parenchymal microglia and hematogenous macrophages invading into the injured tissue. Furthermore, a third population of macrophages exists in the brain (Graeber et al., 1989). Perivascular macrophages are able to quickly phagocyte particles from the cerebrospinal fluid, form a population which undergo rapid turnover with hematogenous macrophages and might be in a position to significantly contribute to the central nervous system immune surveillance (Bechmann et al., 2004). We previously identified few ramified, nonphagocytic cells both of resident and hematogenous origin at all time points following ischemia (Schilling et al., 2003). Unfortunately, our method does not allow to answer the interesting question of how many phagocytes are required to dispose of what amount of damaged neurons. Nevertheless, our data indicate that microglia are more effective in tissue clearance after ischemic damage compared to macrophages recruited from the circulation, and outline a different role for these cell types after cerebral ischemia.

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