Endogenous and Exogenous Inflammatory Cell Responses Following Transient Ischaemia in Mice

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Introduction

Acute cerebral ischaemia induces an inflammatory response in the brain that involves activation of resident microglia and invasion of blood-borne inflammatory cells, such as macrophages, monocytes and granulocytes. The temporal relationship of this inflammatory cell activity in post-ischaemic brain has been extensively studied (1). Recent studies have shown that dextran-coated iron-oxide particles (such as AMI-227) can be used as MRI contrast agents for macrophage labelling *in vivo*. Rausch et al. (2) and Schroeter et al. (3) have shown that AMI-227 positive macrophages are readily detectable in the rat brain following acute stroke. Interestingly, Weber et al. (4) report that, in the subchronic phase of ischaemic infarction, iron-containing native macrophages may become detectable by T_2* MRI. These observations indicate that MRI could be used as a tool to image the cellular nature of the inflammatory response after ischaemia. In this study we investigated the activation of microglial cells and invasion of macrophages and neutrophils following transient focal ischaemia in mice. Permeability of the blood-brain-barrier (BBB) was determined by sequential Gd-DTPA injections and AMI-227 was injected for *in vivo* labelling of macrophages.

Methods

Male C57/BL6 mice (Harlan Olac, UK; n=38) were exposed to middle cerebral artery occlusion (MCAo) for 30 mins during isoflurane anaesthesia (1% in 30% O_2/70% N_2O) as described previously (5), except that laser-doppler was not used. At time of reperfusion AMI-227 (160 µmol of Fe/kg) and Magnevist (0.1 mmol/kg) were injected into the tail vein. Mice were re-anaesthetised after 4h and underwent MRI scanning using a horizontal 7 T SMIS system (Guildford, Surrey, UK). A single loop surface coil (3cm in diameter) was used for both signal reception and transmission. Ten 0.8 mm tick coronal slices (slice gap 0.8 mm) were selected to cover the forebrain. T_1 images were acquired with TR=700ms, TE=10ms, FOV=30mm, 256x128 (zerofilled to 256x256 prior FT). For T_2 images TR was 1500ms and TE either 30 or 60ms. Diffusion images were obtained with TR=1800ms, TE=60ms and b-value=1000 s/mm^2 diffusion weighting applied along the slice selection gradient, data matrix 128x128. Mice were re-injected with AMI-227/Magnevist 24, 48 and 72h after MCAo occlusion, anaesthetised with isoflurane and scanned for MRI as described above. Animals were transcardially perfused with 4% PFA after the last scan, and the brains subsequently frozen. Serial sections were cut on a sliding microtome and stored in cryoprotectant solution at -20°C. Iron oxide particles were detected with Prussian Blue staining with or without subsequent DAB enhancement combined with Silver/Gold intensification (3). Immunofluorescence was performed on free-floating sections. After blocking with normal serum, primary antibodies [rabbit anti-iba-1 (Wako), rat anti-mouse CD45 (Serotec), rat anti-mouse NIMP (Cell Sciences), rat anti-BrdU (Serotec)] were incubated overnight at 4°C. Secondary antibodies were raised in donkey, conjugated with Alexa Fluor 594 or Alexa Fluor 350 (Molecular Probes) dyes. Tomato lectin (Sigma, 6µg/ml) was incubated for 2h at room temperature after biotin blocking and visualized with streptavidin Alexa Fluor 350 conjugate.

Results

Evolutions of T_2 and diffusion lesion volumes are shown in Figs.1A & 1B. In two out of seven mice scanned, the lesion volume extended to the cerebral cortex by 24 h. T_1-weighted images showed no contrast enhancement 4h after MCAo, but strong signal enhancement was evident in all mice beyond 24h due to Gd-DTPA leakage into brain tissue as a sign of BBB damage (Fig. 1C). T_2-weighted images (TE either 30 or 60ms) following AMI-227 injections showed no evidence for signal void in brain parenchyma peculiar to iron-oxide deposits in any time points up to 72h. These data indicate no MRI detectable AMI-227 accumulation into the brain post MCAo.

Iron-laden cells were detectable with straight Prussian Blue staining in spleen sections (Fig.2B) and bone marrow/blood smears (Fig.2A). In contrast, only very few Prussian Blue positive cells (in one out of five animals) were present in the brain parenchyma of the ipsilateral hemisphere 72h after MCAo. DAB and Silver/Gold intensification revealed scattered iron-containing cells in the meninges, in the ischaemic striatum (Fig. 2E) in two out of five animals and in the ipsilateral cortex (Fig. 2D) in two out of five animals (average of 0-3 cells/section) by 72h of reperfusion. Immunofluorescent analysis of brain sections revealed activation of resident microglia in response to focal ischaemia (Fig. 2I). Activated microglia exhibited intensive proliferation (Fig.2J) and phagocytosis of neutrophils was observed (Fig.2K).

Conclusions

The present results show no significant infiltration of blood-borne macrophages to the brain after MCAo in mice during the first three days after the insult. Instead, activation of resident microglial cells is prominent, consistent with a recent study in the mouse MCAo model (6). Meanwhile, BBB leakage is evident along with maturation of ischaemic infarction. These results suggest that invading macrophages are not necessarily contributing to the development of infarction following focal ischaemia with reperfusion. In fact, our findings suggest a possible advantageous role of resident microglia in post-ischaemic brain due to phagocytosis of neutrophils by microglial cells. These data also indicate that, when using AMI-227 as a macrophage contrast agent, one has to be able to exclude direct access of iron-oxide particles to the brain parenchyma through a leaky BBB (7).

Acknowledgements

Supported by Marie-Curie Programme of the European Union, MRC and the Wellcome Trust.

References

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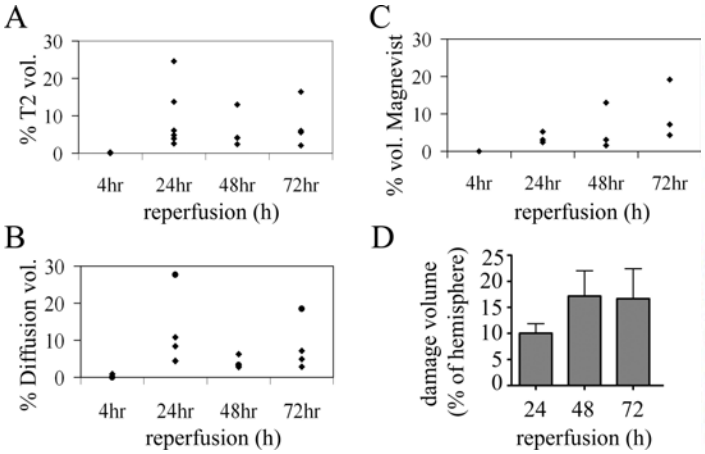

Fig. 1. Graphs show the volume of the affected areas in the ischaemic brains on T2 –(**A**), Diffusion –(**B**) weighted images and on T1 (**C**) with Magnevist, as the percentage of hemisphere measured in individual mice. The volume of ischaemic damage was calculated on cresyl violet-stained brain sections as a percentage of hemisphere (**D**).

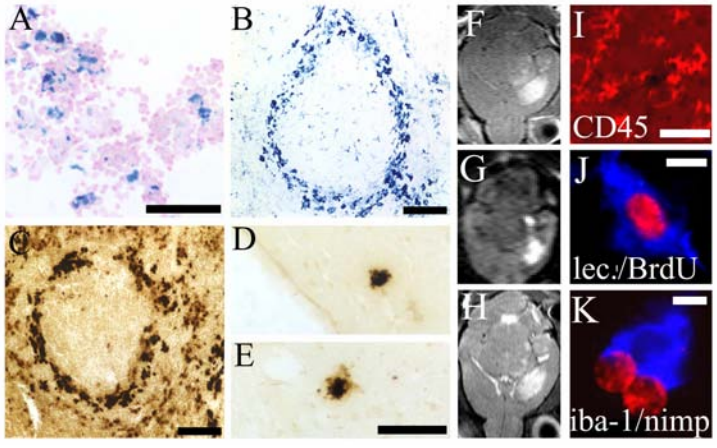

Fig. 2. Prussian Blue staining reveals iron-laden cells on blood leukocyte smears (**A**) and in the spleen (**B**). DAB and Silver/Gold intensification results in improved detectibility of iron in the spleen (**C**) and reveals a few iron-containing cells in the ipsilateral cortex (**D**) and striatum (**E**) at 72h. BBB breakdown and oedema are shown on T1- (F), Diffusion (G) – and T2 (H)–weighted MR images at 72h. Activated microglia in the ischaemic striatum express CD45 (I), lectin (J, blue) and iba-1 (K, blue), incorporate BrdU (J, red) and phagocytose neutrophils (K, red). Scale bars: 100µm (B,C) 50µm (A,D,E,I) and10µm (J,K).