Imaging seizure-induced inflammation using an antibody targeted iron oxide contrast agent

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Early inflammation following status epilepticus has been implicated in the development of epilepsy and the evolution of brain injury, yet its precise role remains unclear. The development of non-invasive imaging markers of inflammation would enable researchers to test this hypothesis in vivo and study its temporal progression in relation to epileptogenic insults. In this study we have investigated the potential of a targeted magnetic resonance imaging contrast agent – vascular cell adhesion molecule 1 antibody labelled iron oxide – to image the inflammatory process following status epilepticus in the rat lithium-pilocarpine model. Intravascular administration of the targeted contrast agent was performed at approximately 1 day following status epilepticus. The control group received diazepam prior to pilocarpine to prevent status epilepticus. Magnetic resonance imaging of rats was performed before and after contrast administration. Comparison with quantitative T2 measurements was also performed. At the end of the study, brains were removed for ex vivo magnetic resonance imaging and histology. Marked focal hypointensities caused by contrast agent binding were observed on in vivo magnetic resonance images in the post status epilepticus group. In particular these occurred in the periventricular organs, the hippocampus and the cerebral cortex. Relatively little contrast agent binding was observed in the control group. T2 relaxation times were not significantly increased for the hippocampus or the cerebral cortex in post status epilepticus animals. These results demonstrate the feasibility of in vivo imaging of seizure-induced inflammation in an animal model of epilepsy. The antibody targeted MRI contrast agent identified regions of acute inflammation following status epilepticus and may provide an early marker of brain injury. This technique could be used to determine the role of inflammation in models of epileptogenesis and to study the potential for anti-inflammatory therapeutic interventions.

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Introduction

There is mounting evidence to suggest that inflammation may be involved in seizure generation (ictogenesis) and the development of some partial epilepsies (epileptogenesis) (Ravizza et al., 2011). The mechanisms through which this occurs are becoming clearer and this knowledge is likely to lead to novel therapeutic approaches. Ictogenesis appears to be exacerbated by inflammatory cytokines, in particular interleukin-1 beta (IL-1β) (Dube et al., 2005; Rodgers et al., 2009) and tumour necrosis factor-alpha (TNF-α) (Riazi et al., 2008) which increase cellular hyperexcitability, possibly via enhanced NMDA-dependent Ca\textsuperscript{2+} influx into neurons (Viviani et al., 2003). Alongside this, it has been shown that blood brain barrier (BBB) disruption can induce epileptiform activity (Marchi et al., 2007a), the mechanism of which is thought to occur via astrocytic uptake of serum albumin resulting in impaired buffering of extracellular potassium (Ivens et al., 2007). Given this evidence, it has been hypothesised that inflammation could provide a crucial mechanism for the development of some partial epilepsies (Vezzani et al., 2011; Yang et al., 2010).

Evidence that inflammation contributes to epileptogenesis was demonstrated by blocking leukocyte adhesion using alpha-4 specific monoclonal antibodies following status epilepticus (SE). This reduced the frequency and severity of seizures in the chronic epilepsy period of the pilocarpine model (Fabene et al., 2008). Furthermore,
administering the cyclooxygenase-2 (COX-2) inhibitor celecoxib following SE reduces the frequency of spontaneous recurrent seizures (SRS) in the pilocarpine model (Jung et al., 2006). Other work has shown that interactions between the innate and adaptive immune system may contribute to both seizure suppression and neuroprotective effects (Zattoni et al., 2011). The COX-2 inhibitor parecoxib did not alter the frequency or duration of SRS when administered following pilocarpine induced SE (Polascheck et al., 2010) and COX-2 inhibitors have been ineffective at preventing the development of epilepsy in electrically induced status epilepticus (Holtman et al., 2009). Taken together, this highlights the need to monitor the inflammatory process in vivo in order to provide greater understanding of the role of inflammation in epileptogenesis, as this could lead to the development of anti-inflammatory therapies as an anti-epileptogenic strategy (Kleen and Holmes, 2010).

Non-invasive imaging of inflammation would enable tracking of the temporal progression of inflammation following epileptogenic insults. Regional cerebral inflammation has previously been imaged in vivo using a novel intra-vascular magnetic resonance imaging (MRI) contrast agent, vascular cell adhesion molecule 1 (VCAM-1) conjugated to micron-sized iron oxide particles (McAteer et al., 2007), which is ideally suited as an imaging agent due to the low background expression of VCAM-1 in normal tissue and a large payload of iron; Akhtar et al., 2010; Hoyte et al., 2010). It is known that VCAM-1 is expressed on the walls of blood vessels and that it mediates the rolling and extravasation of leukocytes across the endothelium (Elcès et al., 1990). As leukocyte adhesion is strongly correlated to BBB permeability (Stokes and Granger, 2000), we hypothesise that VCAM-1 expression occurs prior to vasogenic oedema and the associated MRI T2 relaxation time changes (Choy et al., 2010a; Fabene et al., 2003). Imaging VCAM-1 expression could therefore provide an early and more sensitive marker of inflammation or subtle alterations in blood brain barrier permeability that cannot be detected clinically using gadolinium based contrast agents or T2 weighted MRI (Hoyte et al., 2010). In this study we have imaged seizure induced endothelial activation using micron-sized particles of iron oxide conjugated to VCAM-1 antibodies (VCAM-MPIO) soon after termination of lithium–pilocarpine induced status epilepticus in rats.

### Materials and Methods

The contrast agent (VCAM-MPIO) was synthesised as described in the literature (McAteer et al., 2007). Briefly, (12.5 mg) 1 μm diameter tosylactivated Dynabeads (26% iron content, Invitrogen) were conjugated to (500 μg) monoclonal antibodies specific to rat VCAM-1 (ebioscience, San Diego, CA). IgG-MPIO was synthesised using the same method, except an isotype matched control antibody (Southern Biotech, Birmingham, AL) was used in place of the VCAM-1 antibody. The VCAM-MPIO contrast agent was then used in two different experiments.

**In vitro experiment**

The first experiment was carried out in cell culture to establish whether the contrast agent was bound in vitro in the immortalised rat brain endothelial cell line (GPNT) in the presence of an inflammatory insult. The immortalised rat brain endothelial cell line (GPNT) was generously provided by Prof. John Greenwood, Institute of Ophthalmology, University College London (UCL). GPNT cells were cultured until confluent in 35 mm diameter well dishes. Cells were treated with 10 ng/ml human recombinant TNF-α (Invitrogen, UK) (n = 3) or saline (n = 3) in controls and incubated at 37 °C for 20 h. VCAM-MPIO was added at a concentration of 0.06 mg/ml media and cells were incubated for 2 h at 37 °C then washed several times with PBS (pH 7.4). Quantification of contrast agent binding was performed on grayscale images by thresholding at an arbitrary value. Data is displayed as mean ± SD.

**In vivo experiment**

All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) 1986 Act and institutional ethics regulations. Adult male Sprague–Dawley rats weighing 180–230 g (n = 12) were obtained from the breeding colony of the University College London (UCL) animal facility. All rats were housed in a controlled temperature and humidity environment with a 12 h light/dark cycle with food and water provided ad libitum. Animals were separated into three groups; lithium–pilocarpine control group which did not undergo status epilepticus given VCAM-MPIO contrast (ControlVCAM) (n = 4), lithium–pilocarpine induced status epilepticus with IgG-MPIO isotype matched contrast agent to control for non-specific binding and leakage through the blood brain barrier (SEx1) (n = 3) and lithium–pilocarpine induced status epilepticus with VCAM-MPIO contrast (SExVCAM) (n = 5). It has been previously shown that lithium and pilocarpine may directly cause peripheral inflammation (Marchi et al., 2007b), and could conceivably also directly lead to central nervous system (CNS) inflammation. To control for this effect, the control group received diazepam injections prior to the injection of lithium and pilocarpine (Fig. 1). Injection of diazepam prior to the injection of pilocarpine has shown to completely prevent the development of SE (Turski et al., 1983). In the SExVCAM group, animals were treated with lithium chloride (3 mEq/kg (per kilogramme of body weight), i.p., Sigma-Aldrich, Dorset, UK) 2 h prior to methylsoprolamine (5 mg/kg, i.p., Sigma-Aldrich) injection. Methylisoprolamine was administered to reduce the peripheral effects of pilocarpine. This was followed 15–20 min later by administration of pilocarpine hydrochloride (30 mg/kg, i.p., Sigma-Aldrich) in order to induce status epilepticus. Animals were behaviourally assessed on using the Racine scale (Racine, 1972). The onset of status epilepticus was defined as stage 3 on the Racine scale. Diazepam (10 mg/kg, i.p.) was administered 90 min after the onset of SE to terminate the seizure. Further injections of diazepam were administered as required. The ControlVCAM group received a lithium chloride injection (3 mEq/kg, i.p., Sigma-Aldrich, Dorset, UK), a subsequent injection of diazepam (30 min prior to pilocarpine injection) to prevent SE onset (Turski et al., 1983), followed by methylisoprolamine (20 min prior to pilocarpine injection) and pilocarpine (30 mg/kg).

**In vivo MRI**

MRI was performed using a 9.4 Tesla DirectDrive VNMRS horizontal bore system with a shielded gradient system (Agilent technologies, Palo Alto, CA) and a 4-channel rat head phased-array coil (Rapid Biomedical GmbH, Würzburg, Germany). MRI was performed before and after injection of the contrast agent. Animals were anaesthetised with 4% isoflurane and maintained at 1.5% isoflurane in pure oxygen (1 L/min) throughout the imaging protocol. A physiological monitoring system (SA Instruments, Stony Brook, NY) was used to monitor respiration rate and rectal temperature. Temperature was maintained at 37 ± 0.5 °C using an air warming system. VCAM-MPIO (4 mg of iron/kg) was injected via the right external jugular vein 21.9 ± 0.2 h post-SE onset or 22.1 ± 0.2 h (mean ± SEM) post-pilocarpine administration. Animals were imaged 1 h post-injection to allow time for MPIO clearance. The imaging parameters were as follows: three-dimensional (3D) spoiled gradient-echo (SPGR), TR = 100 ms, TE = 6.5 ms, FA = 30°, matrix = 192 × 192 × 128, FOV = 22 × 22 × 20 mm, acquisition time = 40 min. The images were zero filled to 256 × 256 × 256, giving a final voxel size of 86 × 86 × 78 μm³. T2 measurements were performed before injection of the contrast agent with a multislice multi-echo spin-echo sequence across 13 contiguous slices; TR = 2 s TE = 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88, 96, 104, 112, 120 ms, matrix = 128 × 128, FOV = 25 × 25 mm slice thickness = 1 mm, acquisition time = 4 min.
Approximately 4 h after contrast agent injection, animals were perfused-fixed with 50 ml phosphate buffered saline (PBS, pH 7.4) followed by 50 ml paraformaldehyde (PFA) (4% in PBS, pH 7.4) via a cannula inserted into the left ventricle. The brains were removed and immersed in PFA (4%) overnight. The fixed-brains were immersed in Fomblin (type PFS-1, Solvay Solexis S.p.A., Bollate, Italy) for ex-vivo MRI using a 26 mm diameter birdcage coil (Rapid Biomedical GmbH). The imaging parameters were as follows: 3D SPGR, TR = 350 ms, TE = 9.8 ms, FA = 60°, matrix = 300 × 300 × 300, FOV = 20 × 20 × 20 mm, total acquisition time = 8 h 45 min. The images were zero-filled to 512 × 512 × 300. The data was then reconstructed to 512 × 512 × 150 giving a final voxel size of 39 × 39 × 133 μm³.

**Segmentation of contrast volume and T2 analysis**

The brain was manually segmented on 3D SPGR images on 40 contiguous slices. Regions were classified as hypointense using an automated segmentation programme written in MATLAB (MathWorks, Natick, MA). This involved using a local thresholding approach to account for variations in surface coil sensitivity. This was implemented by firstly scaling the intensity values to between zero and one by dividing by the maximum signal intensity value and then thresholding regions that were less than the local median-C, where C was arbitrarily chosen to be 0.1. The local median was calculated using a 3D median filter with a radius of 5 voxels. Nearest neighbour connectivity was then determined in 3 dimensions (objects were defined as connected if they shared at least one vertex) on the thresholded data and objects were discarded if the object size was below an arbitrary threshold of 4 voxels as these were likely to be due to noise. Quantitative T2 measurements were performed using the multislice multi-echo spin-echo sequence data by calculating power images (Miller and Joseph, 1993) and fitting a single exponential decay to regions of interest (ROIs) using non-linear least squares methods in MATLAB. The first two echo times were excluded from the analysis as this minimises errors resulting from stimulated echoes and produces a better fit to a mono-exponential function. For both datasets ROIs were drawn by an observer blinded to the animal groupings over 4 different brain regions including the dorsal hippocampus, cerebral cortex, thalamus and piriform cortex using a standard rat brain atlas (Paxinos, 2007).

**3D reconstruction**

Three-dimensional reconstruction was performed on a separate in vivo dataset acquired 2 h post contrast administration. This dataset was used as higher signal-to-noise ratio (SNR) facilitated automated brain masking based on thresholding, pixel connectivity and slice-to-slice overlap (Brummer et al., 1993). Hypointense regions were segmented using the same algorithm as above. The imaging parameters were as follows: three-dimensional (3D) spoiled gradient-echo (SPGR), TR = 100 ms, TE = 11 ms, FA = 15°, matrix = 192 × 192 × 128, FOV = 25 × 25 × 20 mm, acquisition time = 40 min. The images were zero filled to 256 × 256 × 192.

**Histology and immunohistochemistry**

Following the perfusion fixation protocol, brains were fixed in PFA (4%) overnight and cryoprotected in 30% sucrose for 24 h then frozen by immersion in dry ice-isopropanol. Brains were sectioned at 30 μm slice thickness using a cryostat (Bright Instruments, Huntington, United Kingdom), fixed in acetone at 4 °C for 20 min and stained for VCAM-1 using a biotinylated primary antibody specific to VCAM-1 (eBioscience) and streptavidin conjugated to horseradish peroxidise (Biolegend, San Diego, CA). The presence of HRP was visualised using diaminobenzidine (DAB) (Thermo Scientific, UK). As a negative control, the primary antibody was omitted. Sections were then counterstained with cresyl violet (0.1%).

**Statistical analysis**

Unless otherwise stated, SEVCAM was compared to the two control groups using Wilcoxon rank-sum tests and Kruskal–Wallis tests. Statistical significance was assigned at p < 0.05. Data is displayed as mean ± SEM.

**Results**

**In vitro**

The rat brain endothelial cell line (GPNT) is known to express VCAM-1 when treated with TNF-α (Romero et al., 2000). After being treated with TNF-α, GPNT cells showed strong VCAM-MPIO binding (Figs. 2b,c). Very little binding was observed in untreated cells (Figs. 2a,c) demonstrating the binding of the contrast agent to VCAM-1 in vitro (number of thresholded pixels = 166,000 ± 21,900 vs. 11,900 ± 3800) (p = 0.02 (t-test)).

**In vivo**

All animals in the SEVCAM group and the SEIgG group progressed to status epilepticus. All animals displayed akinesia and facial automatisms which progressed to tonic–clonic seizures and status epilepticus within 60 min of pilocarpine administration. None of the animals in the control group displayed any signs of behavioural
seizures. Mean Racine seizure scores (n=3) following SE onset were:
0–30 min = 3.2 ± 0.2, 30–60 min = 4.1 ± 0.2, 60–90 min = 4.7 ± 0.4.

Superparamagnetic iron oxide particles, when placed in a strong magnetic field, acquire a different magnetisation to brain tissue. This leads to enhanced relaxation of transverse magnetisation and manifests itself as hypointense regions on gradient echo MRI images. Hypointense regions caused by VCAM-MPIO binding are clearly present on in vivo MRI images in the SEVCAM group and appear to be maximal in the choroid plexus and the periventricular organs (PVO) (Fig. 3c). Additionally, hypointense regions also appear to be present predominantly in the hippocampus and the cerebral cortex on in vivo images. Hypointensities were not identified in the pre-injection images. A similar distribution was observed on high resolution ex vivo 3D SPGR images, corroborating that these hypointensities are caused by VCAM contrast agent in similar brain regions. The ControlVCAM and SEIgG groups showed very few hypointensities on MR images on both

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**Fig. 2.** In vitro binding of VCAM-MPIO to rat brain endothelial cell line. Bright field microscopy at ×400 magnification of rat brain endothelial cell line. 1 μm diameter iron oxide particles can be visualised as dark circular regions on bright field light microscopy (black arrow). (a) GPNT cells without TNF-α treatment, incubated with VCAM-MPIO for 1 h and washed several times (n=3). Very few particles could be observed binding to untreated cells. (b) GPNT cells treated with TNF-α for 20 h in order to induce VCAM-1 expression, labelled and washed in the same manner as panel a (n=3) (magnification ×400, scale bar = 10 μm). (c) Quantitation of iron oxide binding using thresholding.

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**Fig. 3.** 3D SPGR images pre-contrast, post-contrast and ex vivo. Single coronal MR image slices of 78 μm thickness at approximately 3.8 mm posterior from the bregma are shown for a representative animal from each group. Images were acquired 23 h after SE or pilocarpine administration, approximately 1 h after contrast agent administration and ex vivo images were acquired at the end of the study. (a) SEVCAM group rat receiving 4 mg iron per kg VCAM-MPIO. Focal hypointense regions can be observed in the periventricular organs, hippocampus and cerebral cortex both in vivo and ex vivo following contrast agent administration. (b) ControlVCAM group which did not undergo status epilepticus injected as in panel a, demonstrating an absence of contrast agent binding. (c) SEIgG group administered with a control contrast agent IgG-MPIO as in panel a. An absence of contrast agent demonstrates that there was little non-specific binding or leakage through the impaired blood brain barrier.
in vivo and ex vivo images, demonstrating that VCAM-1 expression was seizure induced and also that there is little non-specific binding or leakage across an impaired BBB (Figs. 3a and b).

Quantitation of the inflammatory response

Significantly more hypointense volume was observed in the hippocampus SE VCAM group compared to the Control VCAM group and the SE IgG control (Fig. 4b) (7.0 ± 2.2% vs. 0.5 ± 0.3% and 0.5 ± 0.2%) (p = 0.02). The volume of hypointensity measured in the cerebral cortex was also significantly greater in the SE VCAM group compared to control groups (10.5 ± 4.6% vs. 1.1 ± 0.6% and 1.2 ± 0.5%) (p = 0.02). Hypointense volumes in the thalamus were much less marked than in the cerebral cortex or the hippocampus (2.9 ± 1.6% vs. 0.2 ± 0.09% and 0.5% ± 0.2%) (p = 0.02). Three-dimensional reconstructions display this distribution of contrast agent binding (Fig. 5). Small amounts of hypointensity can be observed in the SE IgG group in the regions surrounding the ventricles and in large blood vessels (Fig. 5), this is likely to reflect the limitations in distinguishing between the presence of contrast agent and the differences in magnetic susceptibility in the normal brain (particularly at high field strength), which usually present at air–tissue, tissue–cerebrospinal fluid interfaces and large blood vessels. T2 measurements were not significantly elevated in the SE group compared to the control group in the hippocampus, cerebral cortex, or the thalamus (Fig. 4a). T2 values were higher in the piriform cortex of the SE VCAM group compared to the Control VCAM group (81 ± 6.2 ms vs. 55 ± 0.41 ms) (p = 0.02).

Immunohistochemistry

VCAM-1 expression in the post SE groups was verified by immunohistochemistry (Figs. 6a, c). VCAM-1 expression appeared to be limited to the vascular endothelium (Figs. 6a, c black arrows) and was present in the hippocampus (Fig. 6c). Very little staining was observed in the control group which did not undergo status epilepticus (Fig. 6b).
shown in the pilocarpine model that COX-2 is upregulated primarily in the hippocampus and piriform cortex whilst expression of other mediators such as Toll-like receptor 2 (TLR2) and TNF-α have been found to be more global (Turrin and Rivest, 2004). In the focal kainate model, inflammatory markers such as upregulation of ICAM-1, microglia activation and BBB disruption occur primarily in the ipsilateral (kainic acid injected) hippocampus (Zattoni et al., 2011). This suggests that, seizure activity leads to the regional expression of inflammatory markers. The cause of this regional expression is likely to be complex and several factors might be involved, including seizure induced release of HMGB1 (Maroso et al., 2010), necrosis, apoptosis and/or ischemia.

MRI T2 changes have been observed as early as 6 h following SE in the severely affected piriform and entorhinal cortices (Andre et al., 2007). Hyperintensities in the hippocampus are generally observed at a slightly later time point, with T2 measurements peaking at about 48 h following SE in rodents (Choy et al., 2010a; Fabene et al., 2003) and within 48 h in humans (Scott et al., 2002). It has also been shown that T2 is predictive of neuronal injury following SE (Choy et al., 2010a). However in the current study we observed no significant increase in T2 in the hippocampus at 22 h following SE, yet marked binding of the targeted contrast agent. This suggests that imaging inflammation using targeted iron oxide contrast agents could provide an early and more sensitive marker of brain injury. There is currently no validated biomarker that predicts the development of epilepsy (Mishra et al., 2011) and a molecular imaging approach, such as reported here, could be used for this purpose.

The intra-vascular nature of the contrast agent used in this study may mean that iron oxide induced contrast is dependent on the blood vessel density within voxels, which is a possible limitation. Furthermore, distinguishing between hypointensities caused by tissue interfaces and those caused by iron oxide based contrast agents is problematic based on automated segmentation algorithms.

Recently it has been hypothesised that the post-seizure inflammatory processes in these regions could contribute to both cell death and hyperexcitability (Vezzani et al., 2011). Studies in epilepsy patients have indicated increased numbers of leukocytes in the brain parenchyma compared to controls (Fabene et al., 2008). However current therapeutic strategies are only targeted towards treating neuronal excitability using anti-epileptic drugs that do not treat the possible detrimental effects of the inflammatory component of the condition, which could lead to both seizure activity and neuronal degeneration. Imaging inflammation in epilepsy patients using targeted iron oxide contrast agents could therefore help determine which subtypes of patients might benefit from anti-inflammatory therapies.

**Conclusions**

The precise role of neuroinflammation in the progression of SE associated injury is unknown. There is therefore a need for more specific imaging markers of inflammation. In this study we have demonstrated in vivo imaging of acute inflammation following pilocarpine induced status epilepticus. Imaging markers of inflammation in models of epilepsy could enable pharmacological modulation and may therefore be used to determine the role of inflammation in epileptogenesis and neuronal injury. Furthermore, imaging of inflammation in clinic could help determine which patients might benefit from anti-inflammatory therapy.

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