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The influence of neuropathology on brain inflammation in human and experimental temporal lobe epilepsy

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ABSTRACT

It is unclear to what extent neuropathological changes contribute to brain inflammation observed in temporal lobe epilepsy (TLE). Here, we compared cytokine levels between histopathologically-confirmed sclerotic hippocampi and histopathologically-confirmed normal hippocampi from TLE patients. We analyzed a similar cytokine panel in the hippocampi of amygdala-kindled rats and we evaluated neuropathological changes by immunohistochemistry. In TLE patients, cytokine levels were not significantly different between sclerotic and non-sclerotic hippocampi. Though kindling resulted in increased astrocyte activation, cytokine levels and microglia activation were unchanged. These results suggest that the chronic epileptic state in TLE can also occur in the absence of intracerebral inflammation.

1. Introduction

Epilepsy is a neurological disorder that affects up to 1% of the world's population (Sander, 2003). Temporal lobe epilepsy (TLE) is the most common type of human epilepsy. One third of TLE patients do not respond to current antiepileptic drugs and this large number of therapy resistant patients calls for the development of novel treatment strategies, which can be developed through a better understanding of the pathophysiology of epilepsy.

The involvement of inflammatory processes in the pathophysiology of epilepsy has received increasing attention over the past years, because several lines of evidence indicate that brain inflammation can contribute to seizure generation: inflammatory mediators can alter neurotransmitter concentration and expression of receptors (Kamikawa et al., 1998; Hu et al., 2000; Bezzi et al., 2001; Stellwagen et al., 2005), alter receptor functioning (Balosso et al., 2008; Maroso et al., 2011), induce functional and structural neuronal network changes (Moynagh et al., 1993), and alter blood–brain barrier (BBB) functioning (Fabene et al., 2008, 2010; Heinemann et al., 2012; Janigro, 2012; Librizzi et al., 2012). Moreover, increased levels of inflammatory mediators have been demonstrated in epileptic brain tissue (Sheng et al., 1994; Crespel et al., 2002; Voutsinos-Porche et al., 2004; Ravizza et al., 2008; Wu et al., 2008; Choi et al., 2009; Varella et al., 2011).

It is unclear to what extent the underlying neuropathological changes contribute to the observed changes in inflammatory proteins. We hypothesize that the presence of neuronal cell loss and gliosis contributes to increased expression of inflammatory proteins in brain tissue, because they are mainly expressed in areas with prominent cell loss in experimental epilepsy (Voutsinos-Porche et al., 2004), and only in TLE patients with cell loss and gliosis, i.e. with hippocampal sclerosis (HS), and not in patients without HS (Crespel et al., 2002; Ravizza et al., 2008). Therefore, we aimed to evaluate whether HS is associated with an inflammatory response by measuring cytokine levels in the hippocampi of TLE patients with and without HS. Secondly, we aimed to determine whether brain inflammation occurs at all when neuronal cell loss is absent. To test this, we evaluated several inflammatory markers in a TLE animal model that lacks extensive neuronal cell loss, i.e. the amygdala kindling (AK) model.

Abbreviations: AK, amygdala kindling; BBB, blood–brain barrier; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; HS, hippocampal sclerosis; IL, interleukin; IL-1ra, IL-1 receptor antagonist; MCP, monocyte chemotactic protein-1; MIP, macrophage inflammatory protein; NeuN, neuronal nuclei; NMDA-R, N-methyl-D-aspartate receptor; PBS, phosphate buffered saline; PSD, postsynaptic density; TLE, temporal lobe epilepsy; TNFα, tumor necrosis factor.

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2. Material and methods

2.1. TLE patients

The hippocampi were collected in the course of surgical treatment for medically refractory TLE at Maastricht University Medical Centre. Informed consent was obtained for the use of brain tissue and access to medical records for research purposes. Extensive pre-surgical evaluation included video-EEG monitoring, neuropsychological testing, MRI, and FDG-PET imaging as needed. Cytokines were analyzed in sclerotic (n = 19, 9 males, mean age ± SEM 44.6 ± 3.54 years) and non-sclerotic hippocampi (n = 4, 3 males, mean age ± SEM 43.4 ± 10.1 years).

2.1.1. Tissue collection

The hippocampi were resected en bloc, immediately cooled for 1 min in 4 °C physiological saline and then cut in two parts perpendicular to the longitudinal axis. One part was fixed in 4% paraformaldehyde overnight at 4 °C, embedded in paraffin, and used for routine histopathological evaluation. Sclerosis was graded according to Wyler et al., ranging from 0 (no HS) to IV (severe HS) (Wyler et al., 1992). The other part was immediately frozen on dry ice and stored at −80 °C until further analysis for a maximum period of 24 months.

2.1.2. Tissue processing and cytokine analysis

Samples were homogenized in Greenberger Lysis Buffer (100 μg: 1 mL) containing 150 mM NaCl, 15 mM Tris, 1 mM $MgCl(H_2O)_6$, 1 mM $CaCl_2(H_2O)_2$, 1% Triton X-100, and a cocktail of protease inhibitors (Roche Diagnostics Nederland B.V., Almere, Netherlands). Concentrations of interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-10, IL-1 receptor antagonist (IL-1ra), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, and tumor necrosis factor alpha (TNFα) were measured simultaneously by a multiplex assay according to the manufacturer's instructions (Millipore, Massachusetts, USA). Concentrations were estimated using a calibration curve obtained from the respective recombinant proteins diluted in lysis buffer with the help of MILLIPLEX analyst software with a five-parameter logistic curve-fitting method. Concentrations were normalized to the total protein concentration of each sample. The lower limit of detection was 3.2 pg/mL for all cytokines and chemokines.

2.2. Animals

Male 10 week old Sprague–Dawley rats, purchased from Harlan (Horst, The Netherlands) were kept under controlled standard conditions (21 ± 2 °C ambient temperature, a 12 hour light/dark cycle, background noise provided by radio, and food and water available ad libitum). The animals adjusted to their housing conditions for one week before surgery. All experimental procedures were approved by the Animal Ethics Committee of Maastricht University and were in accordance with International Standards.

2.2.1. Implantation of kindling electrode

Implantation was performed as described previously (Aalbers et al., 2009; Rijkers et al., 2010). Briefly, rats were operated under general isoflurane anesthesia (5% for induction and 2.5% for maintenance). Additionally, rats received 0.1 mL buprenorfine hydrochloride (Temgesic®, Schering-Plough Inc., Amstelveen, The Netherlands) 30 min before the surgery to reduce perioperative pain. Fifty-two rats were implanted with a custom made electrode that consisted of a bipolar platinum/iridium needle with a 200 μm diameter tip (Department of Instrument Development, Engineering & Evaluation of Maastricht University). This stimulating/recording electrode was implanted in the left basolateral amygdala (coordinates relative to bregma: −2.5 mm posteriorly, 4.8 mm laterally, and 9.6 mm ventrally) using a standard rat stereotact (Dual Manipulator Lab Standard Stereotact, Stoelting Inc., Wood Dale, Ill, USA). In addition, a stainless steel screw was implanted over the nasal sinus that served as reference.

2.2.2. Amygdala kindling

Amygdala stimulation started 10 days after the surgery. Initially stimulation was performed twice daily (first stimulus between 8 and 10 am, second stimulus between 2 and 4 pm; interstimulus interval at least 6 h) with the following stimulation parameters: 2 s, 400 μA, 50 Hz, and 0.2 ms block pulses. A stimulus intensity of 400 μA was chosen to assure that the intensity was above the after discharge threshold for all rats. Stimuli were delivered through a WPI Accupulser A310 connected to a WPI Stimulus Isolation Unit A360 (World Precision Instruments, Sarasota, FL, USA).

All rats were videotaped (Olympus FE-330) during delivery of the kindling stimulus and for as long as the behavioral seizure lasted. Seizure severity was evaluated offline from video-recordings by 2 blinded observers and classified according to the Racine scale (Racine, 1972).

After reaching the fully kindled state, defined as five consecutive stage five seizures, rats received one AK-stimulation per day for two more weeks. Sham rats received an amygdala electrode, which was not stimulated.

2.2.3. (Immuno)histochemistry

Two hours and 24 h after the last seizure, rats (n = 5 per group) received an overdose of pentobarbital (Nembutal, 0.1 mg/kg body weight) and were then trans-cardially perfused with 0.5 M ice cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.5 M PBS. These time points were chosen in order to discriminate acute and chronic seizure effects. Brains were isolated and post-fixed in the same fixative for 90 min and then transferred to 20% sucrose in 0.5 M PBS for 24 h. Subsequently, the brains were frozen by immersion in −40°C isopentane for 3 min and stored at −80°C until immunohistochemistry was performed. Horizontal 40 μm thick sections were serially cut throughout the hippocampus using a cryostat. Standard hematoxylin–eosin (Merck, Germany) staining was used to verify the location of the electrode tip.

Immunohistochemistry was carried out as described previously (Ravizza et al., 2008). Briefly, free-floating sections were successively incubated at 4°C for 30 min in 1 M PBS supplemented with 0.4% Triton X-100 and for 15 min in 1 M PBS supplemented with 3% fetal bovine serum (FBS) and 0.1% Triton X-100. Then sections were incubated overnight at 4°C in 3% FBS in 0.1% Triton X-100 in 1 M PBS and one of the following primary antibodies: mouse anti-glial fibrillary acidic protein as a selective marker for astrocytes (GFAP, diluted 1:2500, Chemicon Int. Inc., Temecula, USA), mouse anti-CD11b as a marker for microglia-like cells (complement receptor type 3, OX-42, diluted 1:100, Serotec Ltd, Oxford, UK), or mouse anti-neuronal specific nuclear protein as a selective neuronal marker (NeuN, diluted 1:1000, Chemicon, USA). Immunoreactivity was tested by the avidin–biotin–peroxidase technique (VECTASTAIN ABC kit, Vector, Burlingame, USA) using 3′,3′-diaminobenzidine (Sigma, Munich, Germany) as chromogen. To avoid interstaining bias, sections from shams and both experimental groups were processed together.

2.2.4. Image analysis

The CA1, CA3, and DG regions were analyzed for each marker in four sections per animal throughout the right ventral hippocampus. Images were captured at 20× magnification using an Olympus AX-70 microscope connected to a digital camera (F-view; Olympus, Tokyo, Japan).

For glial markers, areas of immunoreactivity were highlighted at a constant threshold using NIH ImageJ software (http://rsb.info.nih.gov/ij/). Subsequently, the percentage area above the threshold was measured and the average of the four sections for each animal was calculated (Choi et al., 2009).

Two independent observers estimated neuronal cell loss by counting the amount of NeuN immunoreactive pyramidal cells and dentate