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Does angiogenesis play a role in the establishment of mesial temporal lobe epilepsy?

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ABSTRACT

Mesial temporal lobe epilepsy (MTLE) is a focal epileptic disorder that is frequently associated with hippocampal sclerosis. This study investigated whether blocking angiogenesis prevents the development of seizures and hippocampal atrophy in the pilocarpine rat model of MTLE. To block angiogenesis, a subset of animals were given sunitinib orally. Continuous video recordings were performed to identify seizures. Brains were then extracted and sectioned, and hippocampal surfaces and angiogenesis were assessed. After a latent period of 6.6 ± 2.6 days, the sham-treated pilocarpine rats presented convulsive seizures, while the pilocarpine rats treated with sunitinib did not develop seizures. Sham-treated pilocarpine rats but not sunitinib-treated pilocarpine rats had significantly smaller hippocampi. Endothelial cell counts in sham-treated pilocarpine rats were significantly greater than in controls and sunitinib-treated pilocarpine rats. Blocking angiogenesis immediately following the initial insult in this animal model prevented thus angiogenesis and hippocampal atrophy and averted the development of clinical seizures.

1. Introduction

Epilepsy is one of the most common neurological disorders affecting up to 1% of the population worldwide (Moshé et al., 2015). It is estimated that approximately 50 per 100,000 children develop epilepsy each year (Neubauer et al., 2008). The symptomatic epilepsies represent a significant proportion of epileptic disorders and often develop as a consequence of an initial brain insult such as infection, febrile seizures, vascular accidents, or head trauma (Engel, 1996; Gloor, 1997; French et al., 1993; Salanova et al., 1994). The current pharmacological treatment of epilepsy is based on symptomatic management with anticonvulsant drugs that are ineffective in preventing the complex process of tissue remodeling known as epileptogenesis that follows the initial brain injury and finally leads to chronic epilepsy (Pitkanen and Sutula, 2002; Sutula, 2003). Dissecting the molecular and network mechanisms underlying epileptogenesis may guide the development of disease modifying agents and prevent this chronic condition that is often associated with significant morbidities.

Mesial temporal lobe epilepsy (MTLE) is a significant cause of childhood epilepsy. Among childhood epilepsy, MTLE is the most common symptomatic focal epileptic disorder with onset in late childhood to mid-adolescence. Up to 30% of children are medically intractable, often requiring surgical interventions (Engel, 1996; Wiebe et al., 2011). In MTLE, where seizures originate from within the hippocampus and para-hippocampal structures such as the entorhinal cortex, and/or the temporal neocortex, there is an associated characteristic pattern of brain damage known as Ammon's horn sclerosis or mesial temporal sclerosis (Engel, 1996; Gloor, 1997; Wiebe et al., 2011).

Similar behavioral, electroencephalographic and histopathological findings can be reproduced in laboratory animals. Pharmacologically-induced status epilepticus (SE) with convulsant agents, such as pilocarpine, damages the brain and leads 1–4 weeks later to a chronic condition of recurrent limbic seizures that are poorly controlled by antiepileptic drugs (Chakir et al., 2006; Curia et al., 2008; Löscher and Köhling, 2010) and to hippocampal atrophy

Abbreviations: CA3, cornu ammonis 3; DAPI, 4,6-diamidino-2-phenylindole; DG, dentate gyrus; MTLE, mesial temporal lobe epilepsy; SE, status epilepticus; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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(Curia et al., 2008). Hence, in both humans and in animal models, MTLE is characterized by a seizure-free, latent period during which temporal lobe function becomes progressively altered until the appearance of the first seizure that marks the beginning of the epileptic condition.

Changes in temporal lobe excitability and connectivity, along with cells damages, are presumably triggered by the initial brain insult and represent major players in the epileptogenic processes occurring during the latent period (Pitkanen and Sutula, 2002; Sutula, 2003). However, brain injury also induces the activation of angiogenesis (Shibuya, 2009). Angiogenesis is presumed to have a role in the repair processes following brain injury (Xiong et al., 2010). Increasing evidence suggest that VEGF-mediated angiogenesis may create an adverse hyperexcitable milieu favoring epileptogenesis (Croll et al., 2004; Morin-Brureau et al., 2012; Marchi et al., 2012).

In this pilot study with a small number of animals, we used the pilocarpine rat model of MTLE to explore the effects of blocking angiogenesis on the development of chronic epilepsy and hippocampal atrophy. We hypothesized that blocking angiogenesis immediately following SE might decrease neovascularization within the seizure-onset zones and thus impact the development of chronic epilepsy and hippocampal atrophy in MTLE.

2. Methods

2.1. Pilocarpine rat model of mesial temporal lobe epilepsy

Male Sprague-Dawley rats (250–275 g; $n = 13$) were acquired from Charles River Laboratories (St-Constant, Qc, Canada) and were habituated to the environment for 72 h before pilocarpine treatment. On the day of injection, a subset ($n = 10$) were administered scopolamine methylnitrate (1 mg/kg, intraperitoneal; Sigma–Aldrich, Canada) and 30 min later a single dose of pilocarpine hydrochloride (380 mg/kg, intraperitoneal; Sigma–Aldrich, Canada) (Bortel et al., 2010; Lévesque et al., 2012). The behavior of the rats was scored according to the Racine scale (Racine, 1972); SE was defined as continuous stage 5 seizures. SE was terminated after 1 h by injection of diazepam (5 mg/kg, subcutaneous; CDMV, Canada) and ketamine (50 mg/kg, subcutaneous; CDMV, Canada) (Martin and Kapur, 2008). All experiments were conducted in accordance with the Canadian Council of Animal Care, and were approved by the local animal care committee.

2.2. Treatment blocking angiogenesis

Angiogenesis was blocked pharmacologically with sunitinib, a medication known to target the vascular endothelial growth factor (VEGF) pathway by inhibiting the phosphorylation of the VEGF receptors (VEGFR) (Rodriguez, 2007; Wood, 2012), and for which there is evidence for central nervous system penetration (Patyna and Peng, 2006; Speed et al., 2012; Medioni et al., 2007). Sunitinib maleate 20 mg/kg was given by oral gavage daily to a subset of pilocarpine rats ($n = 4$), starting within two hours after the status epilepticus was terminated and continuing for a total of 14 days. The sham-treated pilocarpine rats ($n = 6$) received a daily oral gavage of saline. Control rats with no pilocarpine or sunitinib treatment ($n = 3$) were also included. The dose of 20 mg/kg of sunitinib maleate used in this study was chosen to reflect a dose that has previously been demonstrated as safe, tolerable and effective (Patyna et al., 2008).

2.3. Evaluation of the latent period and seizure quantification in the chronic phase

After SE, rats were housed individually in custom-made plexiglas boxes ($30 \times 30 \times 40$ cm) and let habituate to the environment for 24 h. Continuous video monitoring (24 h per day) was performed from day 3 to day 21 after SE. For each animal, the latent period was defined as the number of days from the SE until the first spontaneous convulsive seizure was seen on video-monitoring (Biagini et al., 2006; Scorza et al., 2009).

2.4. Hippocampal surface measurements

On day 22 (i.e., 3 weeks after SE), the rats were deeply anesthetized with a an intraperitoneal injection of 0.005 mg/kg xylazine, 0.05 mg/kg ketamine and 0.001 mg/kg acepromazine, and then transcardially perfused with 0.1 M phosphate buffered saline, followed by 4% paraformaldehyde. Brains were extracted and post-fixed in paraformaldehyde overnight at 4 °C, and then they were cryoprotected in 30% sucrose, and serially sectioned into 20 μm coronal sections.

Hematoxyline and eosin staining was performed on two different coronal brain sections of each animal at the level of the hippocampus. Each section was observed under microscope (Leica DM4000B LED) at a magnification of 40×. For each section, overlapping microphotographs were taken using a digital camera attached to the microscope (Leica DFC450C). These were then stitched together using a panoramic image stitching software (Microsoft Research Image Composite Editor) to obtain a picture of the entire sections of the brain. Using an image analysis software (ImageJ) (Image Processing and Analysis in Java) (Schneider et al., 2012) converting the scale of the original pictures in mm^2, the surfaces of each hippocampus and each hemisphere were measured for each animal, and hippocampus to hemisphere ratio was calculated (Garcia-Finana et al., 2006). The mean of the right and the left measurements for each animal was used for the calculations.

2.5. Angiogenesis evaluation

To assess angiogenesis, immunohistochemistry was used to examine microvessel density. Sections were labeled with lectin (biotinylated isolectin B4) (Sigma–Aldrich: L2140, St-Louis, MO, USA) (dilution 20 μg/mL in 0.1 M tris-buffered saline [pH 7.4] and 0.5% Triton X-100, incubation time 2 h at room temperature) (Springer, 2010). To detect lectin binding, sections were incubated with streptavidin Alexa Fluor® 350 conjugate (Molecular Probes®, Life Technologies: S11249, Carlsbad, CA, USA) (dilution 1:300 in tris-buffered saline, incubation time 2 h at room temperature) (Ndode-Ekane et al., 2010). Then, these sections were rinsed with tris-buffered saline and cover slipped with Vectashield Mounting media containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories: H1200, Burlingame, CA, USA) that was used to visualize cell nuclei (Ndode-Ekane et al., 2010; Hallene et al., 2006). The endothelial cells count was assessed as per previously described methods (Hallene et al., 2006; Iwai et al., 2007; Noguchi et al., 2008; Rigau et al., 2007; Wintermark et al., 2013). Cells co-labeled with lectin and DAPI were identified as endothelial cells (Springer, 2010; Hallene et al., 2006). To estimate the density of microvessels, single immunoreactive endothelial cells were counted as individual microvessels; endothelial staining in large vessels with tunica media were disregarded in microvessel counts (Hallene et al., 2006; Iwai et al., 2007; Noguchi et al., 2008; Wintermark et al., 2013). For each animal, two fields of view in three different hippocampal regions of interest (i.e., the area 1 of cornu ammonis [CA1], the area 3 of cornu ammonis [CA3], and the dentate gyrus [DG]) were assessed on two different coronal brain sections at the level of the