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Inflammatory mechanisms contribute to the neurological manifestations of tuberous sclerosis complex

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ABSTRACT

Epilepsy and other neurological deficits are common, disabling manifestations of the genetic disorder, tuberous sclerosis complex (TSC). Brain inflammation has been implicated in contributing to epileptogenesis in acquired epilepsy due to brain injury, but the potential role of inflammatory mechanisms in genetic epilepsies is relatively unexplored. In this study, we investigated activation of inflammatory mediators and tested the effects of anti-inflammatory treatment on epilepsy in the *Tsc1*-GFAP conditional knock-out mouse model of TSC (*Tsc1*^{GFAP}CKO mice). Real-time quantitative RT-PCR, immunohistochemistry, and Western blotting demonstrated increased expression of specific cytokines and chemokines, particularly IL-1β and CXCL10, in the neocortex and hippocampus of *Tsc1*^{GFAP}CKO mice, which was reversed by treatment with a mammalian target of rapamycin complex 1 (mTORC1) inhibitor. Double-labeling immunohistochemical studies indicated that the increased IL-1β was localized primarily to astrocytes. Importantly, the increase in inflammatory markers was also observed in astrocyte culture *in vitro* and at 2 weeks of age in *Tsc1*^{GFAP}CKO mice before the onset of epilepsy *in vivo*, indicating that the inflammatory changes were not secondary to seizures. Epicatechin-3-gallate, an inhibitor of IL-1β and CXCL10, at least partially reversed the elevated cytokine and chemokine levels, reduced seizure frequency, and prolonged survival of *Tsc1*^{GFAP}CKO mice. These findings suggest that mTOR-mediated inflammatory mechanisms may be involved in epileptogenesis in the genetic epilepsy, TSC.

Introduction

Tuberous sclerosis complex (TSC) is a genetic disorder, characterized by tumor or hamartoma formation in multiple organs (Crino et al., 2006; Orlova and Crino, 2010). Neurological involvement often accounts for the most disabling symptoms of TSC, including drug-resistant epilepsy, intellectual disability, and autism (Chu-Shore et al., 2010; Holmes et al., 2007). TSC is caused by mutations in the *TSC1* or *TSC2* genes, which leads to hyperactivation of the mammalian target of rapamycin complex 1 (mTORC1) pathway and stimulates cell growth and proliferation, promoting tumor growth. The use of mTOR inhibitors represents a rational, proven approach for treating tumors in TSC (Franz et al., 2013; Krueger et al., 2010).

Although TSC patients can develop brain tumors, the chronic neurological symptoms of epilepsy, intellectual disability, and autism are generally not directly caused by tumor growth *per se*. Cortical tubers, which represent static, developmental malformations or hamartomas of the brain, may contribute to some of the chronic neurological manifestations of TSC, especially epilepsy. However, there is also accumulating evidence that non-tuber, structurally normal-appearing regions of the brain possess cellular and molecular abnormalities that promote neurological dysfunction (Wong, 2008).

Independent of tumor growth, the mTORC1 pathway has also been implicated in promoting epilepsy and intellectual disability in TSC patients, and mTOR inhibitors are being tested in clinical trials as potential treatments for these neurological symptoms (Krueger et al., 2013). Even if mTOR inhibitors are effective against neurological manifestations of TSC, the critical mechanisms downstream from mTORC1 causing epilepsy and neurocognitive dysfunction in TSC are poorly understood. As mTORC1 inhibitors have significant side effects, such as immunosuppression, identification of these downstream mechanisms may lead to more targeted therapies, with more specific efficacy and fewer side effects.

Brain inflammation has been strongly implicated in the pathophysiology of epilepsy and other neurological disorders (Vezzani et al., 2013a, b; Xu et al., 2013). While activation of inflammatory mechanisms in

Abbreviations: ANOVA, analysis of variance; CKO, conditional knock-out; ECG, epicatechin-3-gallate; EEG, electroencephalography; GFAP, glial fibrillary acidic protein; IL-1 β, interleukin-1β; KO, knock-out; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; RT-PCR, reverse transcriptase polymerase chain reaction; SEGA, subependymal giant cell astrocytoma; TSC, tuberous sclerosis complex.

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response to acquired brain injury is perhaps not surprising, a more novel idea is that brain inflammation could also be important in the pathophysiology of developmental or genetic neurological disorders. In fact, inflammatory markers, such as cytokines and chemokines, have been found in brain specimens from patients with genetic malformations of cortical development, including TSC (Boer et al., 2008, 2010; Maldonado et al., 2003; Prabowo et al., 2013), but the pathophysiological significance of inflammation in TSC is poorly understood. Thus, the purpose of this study is to identify specific inflammatory mechanisms, downstream from mTOR, activated in the brain of a mouse model of TSC and determine the effect of modulating these mechanisms.

Materials and methods

Animals and drug treatment

Care and use of animals were conducted according to an animal protocol approved by the Washington University Animal Studies Committee. *Tsc1*$^{flox/flox}$-GFAP-Cre knock-out (*Tsc1*^{GFAP}CKO) mice with conditional inactivation of the *Tsc1* gene predominantly in glia were generated as described previously (Uhlmann et al., 2002). *Tsc1*$^{flox/+}$-GFAP-Cre and *Tsc1*$^{flox/flox}$ littermates have previously been found to have no abnormal phenotype and were used as control animals in these experiments.

In some experiments, three-week-old *Tsc1*^{GFAP}CKO mice were treated with rapamycin (3 mg/kg/day) or vehicle for one week, and brain tissues were then harvested for Western blot, real-time quantitative RT-PCR, or immunohistochemistry analysis. Rapamycin (LC Labs, Woburn, MA) was initially dissolved in 100% ethanol, stored at −20 °C, and diluted in a vehicle solution containing 5% Tween 80, 5% PEG 400 (Sigma, St. Louis, MO), and 4% ethanol immediately before injection or adding to the culture medium.

In other experiments, three-week-old *Tsc1*^{GFAP}CKO mice were treated with vehicle (saline) or Epicatechin-3-gallate (ECG, Sigma, St. Louis, MO). ECG dissolved in saline was administrated by peritoneal injection at the dose of 12.5 mg/kg/d for one week for Western blot and immunohistochemistry analysis, for four weeks for histology, and for up to 12 weeks for video-EEG monitoring. Vehicle-treated non-KO littermates served as additional controls. Other vehicle or ECG-treated *Tsc1*^{GFAP}CKO mice and control mice were monitored for body and brain weight measurements or for survival analysis.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was used to screen a panel of inflammatory markers (Table S1). Total RNA was prepared from brain of two or four week old *Tsc1*^{GFAP}CKO or control mice, or from cultured astrocytes of *Tsc1*^{GFAP}CKO or control mice. Following DNase I treatment (Invitrogen, Grand Island, NY), cDNA was synthesized using iScript Reverse Transcription Kit (BIO-RAD, Hercules, CA). The following conditions were used for reverse transcription: 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min.

Each 25 μl PCR contained 2 μl cDNA, 12.5 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA), and 12.5 pmol of each primer. Real-time quantitative PCR was performed in 96-well optical reaction plates on the AB1 7000 Real-Time PCR System (Applied Biosystems) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Emitted fluorescence for each reaction was measured at the annealing/extension phase. All oligonucleotide primers used for quantitative PCR were designed using Primer Express v2.0 (Applied Biosystems). Calculated copies were normalized against copies of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Forward and reverse primer sets are listed in Table S1.

Western blotting

Western blot analysis was used to measure protein levels of CXCL10 in the brains of *Tsc1*^{GFAP}CKO mice, using standard methods as described previously (Zeng et al., 2008). Briefly, brains were dissected and homogenized separately. Equal amounts of total protein extract were separated by gel electrophoresis and transferred to nitrocellulose membranes. β-actin was used as a loading control. After incubating with primary antibodies to CXCL10 (1:1000; Abcam, Cambridge, MA), or β-actin (1:2000; Cell Signaling Technology), the membranes were reacted with a peroxidase-conjugated secondary antibody (1:1000, Cell Signaling Technology). Signals were detected by enzyme chemiluminescence (GE Healthcare Life Science, Little Chalfont Buckinghamshire, UK) and quantitatively analyzed with ImageJ software (NIH, Bethesda, MD).

Immunohistochemistry/histology

Histological analysis was performed to assess glial proliferation and neuronal organization by standard methods, as previously described (Zeng et al., 2008). In brief, brains were perfusion-fixed with 4% paraformaldehyde and cut into 45 μm sections with a cryotome. Some sections were stained with 0.5% cresyl violet. Other sections were labeled with GFAP antibody (anti-GFAP, mouse; 1:500; Cell Signaling Technology, Beverly, MA), and then Cy3-conjugated goat anti-mouse IgG (1:500; Jackson Immuno, West Grove, PA).

In other experiments, immunohistochemistry was performed for IL-1β, by labeling with primary antibody (anti-IL-1β, rabbit, 1:500; Abcam), followed by labeling with secondary antibody Alexa-488 conjugated goat anti-rabbit IgG (1:500; Life Technologies, Grand Island, NY). Some sections were double stained with GFAP antibody (anti-GFAP, mouse; 1:500; Cell Signaling Technology), NeuN antibody (anti-NeuN, mouse, 1:500; Millipore, Billerica, MA), or Iba1 antibody (anti-Iba1, mouse, 1:500; Millipore), followed by labeling with secondary antibodies: Alexa-488 conjugated goat anti-rabbit IgG (1:500; Life Technologies, Grand Island, NY) or Cy3 conjugated goat anti-mouse IgG (1:500; Jackson Immuno, West Grove, PA). In addition, some sections were counterstained with TO-PRO-3 Iodide (1:1000; Life Technologies) for the nonspecific nuclear staining of all cells.

For blinded analysis, images were acquired with a Zeiss LSM PASCAL confocal microscope (Zeiss Thornwood, NY), or with a Nanozoomer HT system (Hamamatsu, Bridgewater, NJ). In images from coronal sections at approximately 2 mm posterior to bregma and approximately 1 mm from midline, regions of interest were marked in neocortex by a 200 μm-wide box spanning from the neocortical surface to the bottom of layer VI and in hippocampus by areas up to 0.04 mm^2 within the striatum radiatum of CA1 and dentate gyrus. GFAP-immunoreactive cells were quantified in the regions of interest from two sections per mouse from a total of eight mice per group. Similarly, IL-1β positive cells and TO-PRO-3 Iodide stained cell numbers were counted in the sections of each groups, and IL-1β positive cell numbers were normalized to TO-PRO-3 Iodide stained cell number, and were presented as IL-1β positive cells/100 TO-PRO-3 positive cells.

ELISA of serum IL-1β and CXCL10 expression

IL-1β and CXCL10 proteins were assayed in duplicate from serum of four week old *Tsc1*^{GFAP}CKO mice and control mice with the mouse IL-1β and CXCL10 enzyme-linked immunosorbent assay (ELISA) kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. Additional control mice were treated with lipopolysaccharide (LPS, 250 μg/kg, i.p.), as a positive control. Blood was collected 6 h following i.p. LPS administration and serum was separated from clotted blood overnight at 4 °C.