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Altered transforming growth factor beta/SMAD3 signalling in patients with hippocampal sclerosis

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ABSTRACT

Transforming growth factor beta (TGFβ) signalling cascade has been implicated in enhancing neuronal excitability and excitatory synaptogenesis following blood brain barrier (BBB) damage and inflammation. We aimed to study if TGFβ signalling expression is altered in patients with Hippocampal Sclerosis (HS). We probed into the protein expression level of the ligand transforming growth factor beta 1 (TGFβ1), transforming growth factor beta receptor II (TGFβRII) and downstream signalling molecule SMAD3 and phosphorylated SMAD3 (pSMAD3) on surgically resected hippocampal samples of thirty-four patients with HS through immuno-blotting. The increase in protein expression level of the ligand TGFβ1 was 285 ± 1.15% higher and its receptor TGFβRII was 170 ± 0.98% higher in hippocampus of patients with HS in comparison to the autopsy hippocampal control samples. The expression of the downstream signalling molecules, SMAD3 is 157 ± 0.13% and 106 ± 0.17% higher in patients with HS as compared to both types of non-seizure controls. The expression of active form of SMAD3, pSMAD3 (2.6010 ± 1.2735) was significantly upregulated in hippocampus of patients with HS compared to autopsy hippocampal controls (0.7899 ± 0.3688). While the expression of pSMAD3 (1.527 ± 0.9425) was significantly upregulated in hippocampus of patients with HS with another type of non-seizure control viz. tumour periphery tissue (0.5791 ± 0.2679), hence strongly supporting the altered expression of the pathway. This study provides the first evidence of alteration of TGFβ pathway in patients with HS which could be a potential therapeutic target.

1. Introduction

Hippocampal sclerosis (HS) is the most common form of drug resistant epilepsy (DRE) (Curia et al., 2014) which is a large scale network disorder and involves temporal lobe structures (Bernhardt et al., 2015). It is well established that damage to the blood brain barrier (BBB) and inflammation are the coexisting aspects of epileptogenesis and ictogenesis (Avoli et al., 2002) in HS supported by an array of clinical and experimental data (Aronica and Gorter, 2007; Ravizza et al., 2008). In view of these processes as a common denominator of various events leading to epileptogenesis, a noteworthy pleiotropic cytokine which probably plays a significant role is transforming growth factor beta (TGFβ).

Canonical TGFβ signalling occurs via SMAD dependent pathways. It is mediated mainly by two serine/threonine kinase receptors, transforming growth factor beta receptor I (TGFβRI) and transforming growth factor beta receptor II (TGFβRII), which form a hetero tetrameric complex with the TGFβ1 ligand. The activated TGFβRI recruits and phosphorylates the R-SMAD proteins, SMAD2/3 which then form a heterocomplex with the co-SMAD, SMAD4 (Wrana et al., 1994) to regulate the transcription of the target genes (Massagué et al., 2005). SMAD2 and SMAD3 are highly homologous but they show differential

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target gene binding specificity and transcriptional activity (Liu et al., 2016). Various animal model studies have established that TGFβ signalling promotes epileptogenesis by enhancing neuronal excitability and excitatory synaptogenesis following BBB damage and extravasation of serum albumin into the brain (Weissberg et al., 2015). Levy et al., 2015 showed that SMAD3 blockers cause decrease in severity of seizures emphasizing the role of TGFβ/SMAD3 pathway in seizure generation. Blocking TGF-β receptors also suppressed most albumin induced transcriptional changes in the brain, including inflammatory responses, and reduced epileptiform activity (Bar-Klein et al., 2014). These studies portray the role of TGFβ signalling in epileptogenesis but its role in HS is not clear. In this perspective, we investigated the expression of TGFβ signalling at ligand TGFβ1, receptor TGFβRII and unphophorylated and phosphorylated forms of the downstream signalling molecule SMAD3 levels in patients with HS. We hypothesize that the molecules involved in TGFβ signalling could be altered in patients with HS. We used sclerotic hippocampal tissues resected from patients with HS and two types of non-seizure controls (autopsy hippocampal tissue and tumour periphery tissue) to study protein expression levels of TGFβ1, TGFβRII, SMAD3, pSMAD3.

2. Materials and methods

2.1. Patients

Patients with drug resistant epilepsy (DRE), defined as the failure of adequate trials of two appropriately chosen antiepileptic drug schedules with acceptable side effects underwent pre-surgical evaluation; this included clinical assessment, long term video-electro encephalogram (EEG) monitoring and an epilepsy-protocol magnetic resonance imaging (MRI) [3T], Fluoro-2-deoxyglucose positron emission tomography (FDG-PET), ictal and interictal single photon emission computer tomography and were performed when deemed necessary by the treating epileptologist. Patients' details were discussed at the weekly Epilepsy Surgery case conference attended by Epileptologists, epilepsy surgeons, neuro-radiologists and Nuclear Medicine specialists; suitableness of patients for epilepsy surgery was decided on the basis of concordance of video-EEG, MRI findings and those of other ancillary investigations. Consecutive patients whose MRI showed HS underwent the standard anterior temporal lobectomy (ATL) with amygdalohippocampectomy (AH) and were included in the study as cases. Neuropsychological assessment was done for all cases. The decision for surgical resection was taken after explaining the available options to patients or their legally authorized representatives and obtaining their written informed consent. The tissue sample from the resected hippocampus was used to confirm the diagnosis of HS by the histopathologist.

Cortical tissues from the periphery of gliomas and meningioma in patients without seizures (harvested during planned surgical resection) were used as non-seizure controls, once confirmed to be normal (devoid of tumour) on histopathological examination (Data not shown); hippocampal tissues from people who underwent autopsies and were not known to have any brain pathology were harvested within 12 h of death and used as non-seizure controls (Dixit et al., 2017). The clinical characteristics of the patients are presented in Table 1.

2.2. Ethical statement

Prior to starting the study, approval was granted by the Institutional Ethics Committee (IECPG/-40/27.11.2015, RT-3/30.12.2015), All India Institute of Medical Sciences, New Delhi, India. We declare that we followed the tenants of Declaration of Helsinki in our study.

2.3. Immunodetection

Proteins were isolated from the resected sclerotic hippocampal samples of patients with HS (n = 34) and non-seizure control patients (n = 25) in the radioimmunoprecipitation assay buffer (RIPA) (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, pH 7.5), supplemented with protease inhibitor cocktail and phosphatase inhibitors. Protein concentrations were measured using a Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, USA) and a microplate reader at 570 nm (Microplate Reader (iMark™ Microplate Absorbance Reader, Bio-Rad, USA). In each lane fifty micrograms of protein were loaded on 10% polyacrylamide gels along with Precision Plus protein dual colour marker (Bio-Rad, USA) and transferred onto polyvinylidene fluoride membranes. Blocking of the membrane was done in 5% skimmed milk in Tris buffered saline (200 mM Tris-HCl, 1·37 M sodium chloride, pH 7·6) with 0·1% Tween 20 for 1 h at RT. Primary antibodies including human anti-TGFβ1(1:250), human anti-TGFβRII (1:200), human anti-pSMAD3 (1:400) (Santa Cruz Biotechnology, Inc) and human anti-SMAD3 (1:600; Abcam) were diluted in Tris buffered saline with 0·1% Tween 20 and kept at 4°C overnight and appropriate secondary anti-goat IgG-HRP antibody, 1:1000, Sigma) diluted in Tris buffered saline with 0·1% Tween 20 for 1 h at RT. Antibody labelling was detected with a FluorChem M imager (Cell BioSciences, Santa Clara, CA, USA) using enhanced chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fischer Scientific). Densitometric analysis of the desired band was done by ImageJ software. Protein expression was normalized to the expression of GAPDH (loading control). All densitometries are expressed in arbitrary units (AU).

2.4. Statistical analysis

For immunoblot analysis of TGFβ1, TGFβRII, SMAD3, pSMAD3 expression, statistical significance was tested by Mann Whitney *U* test using SigmaPlot13.1 (Systat Software, Inc., Chicago, IL). Data indicated mean ± SD with n representing number of patients as indicated in the figure legend. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Differential expression of TGFβ1 ligand and TGFβRII receptor expression in hippocampal samples of patients with HS

To explore the altered expression of canonical TGFβ signalling we first analysed the expression of upstream ligand TGFβ1 which directly binds to the downstream TGFβRII receptor (Derynck and Feng, 1997). Western blot analysis of TGFβ1 in resected hippocampal samples of twenty patients with HS and nine autopsy patients revealed significant upregulation of TGFβ1 in patients with HS (2.4258 ± 1.4215; Fig. 1) in comparison to autopsy controls (0.6307 ± 0.2636; Fig. 1). Henceforth we assessed the protein expression levels of TGFβRII in patients with HS. Western blot analysis of TGFβRII in hippocampal samples of fifteen patients with HS (2.417 ± 1.40; Fig. 2) and nine autopsy controls (0.897 ± 0.419; Fig. 2) demonstrated significant upregulation of TGFβRII in patients with HS in comparison to autopsy controls. TGFβ1 showed 285 ± 1.15% and TGFβRII showed 170 ± 0.98% higher expression in the hippocampi of patients with HS when compared to those of the autopsy controls. The densitometric analysis of TGFβ1 and TGFβRII in the hippocampal samples of patients with HS and control were done by quantifying band intensities normalized to GAPDH for individual samples. Blots showed a single band between 50 kDa and 75 kDa for TGFβ1 and TGFβRII and ~37 kDa for GAPDH. Mann Witney test indicated $p < 0.001$ for TGFβ1 and $p < 0.01$ for TGFβRII.

3.2. TGFβRII regulated SMAD3 phosphorylation in hippocampal samples of patients with HS

Western blot analysis of SMAD3, the downstream effector molecule in resected hippocampal samples of ten patients with HS and six