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Elevated expression of pleiotrophin in pilocarpine-induced seizures of immature rats and in pentylenetetrazole-induced hippocampal astrocytes *in vitro*

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ABSTRACT

Pleiotrophin (PTN) is a secreted extracellular matrix (ECM)-associated cytokine that has emerged as an important neuromodulator with multiple neuronal functions. In the present study, we detected and compared the dynamic expression of PTN in the hippocampus and adjacent cortex of immature rats with pilocarpine-induced epilepsy. Moreover, we also confirmed the results by examining PTN expression in hippocampal astrocytes cultured in the presence of pentylenetetrazole (PTZ). Immunohistochemistry showed faint immunostaining of PTN in the control hippocampus and adjacent cortex. Notably, PTN immunoreactivity began to increase in relatively small cells in the hippocampus and adjacent cortex at 2 h and 3 weeks after seizures, and the labeling intensity reached the maximum level in the hippocampus and adjacent cortex at 8 weeks after seizures. Furthermore, we also found that PTZ treatment significantly reduced astrocytic viability in a dose-dependent manner and time-dependently increased expression levels of PTN in hippocampal astrocytes. In conclusion, our data suggest that increased expression of PTN in the brain tissues may be involved in epileptogenesis.

Introduction

Epilepsy is a serious and chronic neurodegenerative disease that occurs frequently during childhood, particularly in the neonatal period (Wirrell, 2005; Friedman and Sharieff, 2006). This neurological disorder is generally characterized by recurrent unprovoked seizures and an imbalance between neuronal excitation and inhibition (Scharfman, 2007). Temporal lobe epilepsy (TLE) is the most common subtype of focal epilepsy and constitutes approximately 30–40% of all epilepsies (Hwang and Hirose, 2012). Despite extensive research efforts, the mechanisms underlying the pathophysiology of epilepsy remain elusive and about 20–40% of all patients are resistant to currently available antiepileptic drugs (Mohanraj and Brodie, 2006; French, 2007). Accumulating evidence indicates that brain inflammation and remodeling of the extracellular matrix (ECM) are key processes in the pathogenesis of epilepsy (Dityatev, 2010; Vezzani et al., 2011). Thus, comparative analysis of cytokine gene expression involved in these processes may be helpful to understand epileptogenesis.

Pleiotrophin (PTN), a secreted ECM-associated cytokine, belongs to a novel two member family of heparin binding molecules that also includes Midkine (Rauvala, 1989). PTN was initially isolated as a growth factor from the bovine uterus and as a neurite outgrowth-promoting factor from the neonatal rat brain (Milner et al., 1989; Merenmies and Rauvala, 1990). Subsequently, PTN has emerged as an important neuromodulator that displays various neuronal functions in the central nervous system, including neuronal survival, differentiation and migration (Wanaka et al., 1993; Silos-Santiago et al., 1996; Maeda and Noda, 1998; Asai et al., 2011). Recent evidence indicates that PTN is involved in a variety of neurological diseases, such as Alzheimer's disease (Wisniewski et al., 1996), cerebral ischemia (Yeh et al., 1998), peripheral nerve injury (Blondet et al., 2005), and Parkinson's disease (Marchionini et al., 2007). However, the potential role of PTN in the pathophysiology of epilepsy has not yet been investigated.

In the present study, we detected the dynamic expression of PTN in the hippocampus and adjacent cortex in an immature rat model of TLE at different time points after seizures induced by lithium chloride-pilocarpine. Furthermore, we also confirmed the results by examining PTN expression in hippocampal astrocytes cultured in the presence of pentylenetetrazole (PTZ).

Abbreviations: TLE, temporal lobe epilepsy; ECM, extracellular matrix; PTN, pleiotrophin; PTZ, pentylenetetrazole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's Modified Eagle's Medium.

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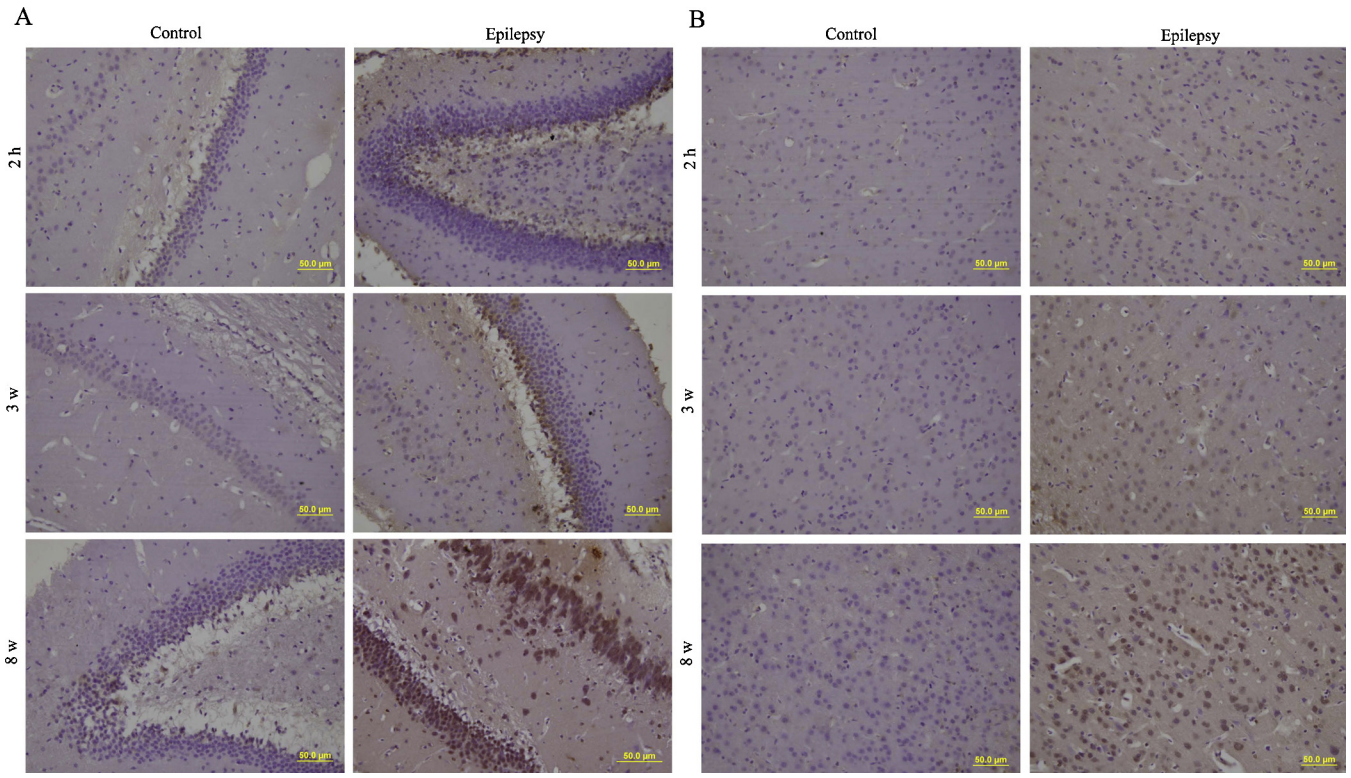

Fig. 1. Immunohistochemistry shows PTN-positive cells in the hippocampus (A) and adjacent cortex (B) of immature rats following pilocarpine administration. Faint immunoreactive staining of PTN is seen in the hippocampus and adjacent cortex of a control rat, while gradual increased immunoreactive staining of PTN was observed in the hippocampus and adjacent cortex of a rat from 2 h to 8 weeks post-seizure. Scale bar = 50 μM.

Materials and methods

Animals and ethics

Immature Sprague-Dawley rats of either sex at postnatal day 11 were obtained from the Experimental Animal Centre of China Medical University. They had free access to food and water and were housed in a temperature-controlled room at 19–22 °C with a 12-h light/dark cycle. This animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University.

Rat model of epilepsy

Forty-eight immature rats were randomly assigned into the control group ($n = 24$) and the epilepsy group ($n = 24$). Epilepsy was induced by lithium chloride and pilocarpine injection. In brief, the rats in the epilepsy group were subjected to an intraperitoneal injection of lithium chloride (127 mg/kg; Sigma–Aldrich, St. Louis, MO, USA) 20 h prior to the administration of pilocarpine (30 mg/kg, i.p., Sigma–Aldrich). Pretreatment with methylscopolamine (1 mg/kg, i.p.; Sigma–Aldrich) 30 min before the first pilocarpine administration was used to reduce peripheral cholinergic effects. Repeated injections of pilocarpine were performed (10 mg/kg, i.p.) every 30 min until onset of *status epilepticus* was observed. The total dose of pilocarpine injection was limited to a maximum of 60 mg/kg per animal. The evoked seizures were classified according to Racine's standard (Racine, 1972). At 90 min after the onset of convulsive seizures at stage 4 or 5, the rats were administered diazepam (10 mg/kg, i.p.; Sigma–Aldrich) to stop the seizure activity. Control animals were treated with saline injections instead of pilocarpine and methylscopolamine. At 2 h, 3 and 8 weeks after seizures, eight rats from each group were sacrificed under deep anesthesia with 10% chloral hydrate (350 mg/kg). The hippocampus and adjacent cortex were rapidly removed for further analysis.

Primary culture of rat astrocytes

Primary rat astrocytes were cultured from the hippocampus of neonatal rats by mechanical dissociation using the procedures as previously described (McCarthy and de Vellis, 1980). The dissociated cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1 mM glutamine at 37 °C in 95% air/5% CO_2. After reaching 80–90% confluence, non-astrocytes, such as microglia, were detached by shaking the flasks. Replacement of medium was then repeated twice a week for the continuation of the culture process. The experiments were initiated after the second passage. For PTZ treatment, astrocytes were incubated with 20 mM PTZ for different intervals and then harvested for quantitative real-time PCR and Western blot analysis.

Immunohistochemistry

Formalin-fixed and paraffin-embedded brain tissues were cut into 5 μm thick sections, deparaffinized in xylene, and rehydrated in serial dilutions of ethanol. After antigen retrieval and blocking of endogenous peroxidase activity, non-specific binding sites were eliminated by incubation in 5% normal goat serum for 1 h. Next, the tissue sections were incubated overnight at 4 °C in a humid chamber with a rabbit polyclonal antibody to PTN (Biosynthesis Biotechnology, Beijing, China), followed by biotinylated goat anti-rabbit IgG antibody and peroxidase-conjugated streptavidin. Finally, sites of bound antibody were visualized with 3,3′-diaminobenzidine, and the immunolabeled sections were