Research Article

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Gli1 activation and protection against hepatic encephalopathy is suppressed by circulating transforming growth factor β1 in mice

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Background & Aims: Hepatic encephalopathy (HE) is a neurologic disorder that develops during liver failure. Few studies exist investigating systemic-central signalling during HE outside of inflammatory signalling. The transcription factor Gli1, which can be modulated by hedgehog signalling or transforming growth factor β1 (TGFβ1) signalling, has been shown to be protective in various neuropathies. We measured Gli1 expression in brain tissues from mice and evaluated how circulating TGFβ1 and canonical hedgehog signalling regulate its activation.

Methods: Mice were injected with azoxymethane (AOM) to induce liver failure and HE in the presence of Gli1 vivo-morpholinos, the hedgehog inhibitor cyclopamine, Smoothened vivo-morpholinos, a Smoothened agonist, or TGFβ-neutralizing antibodies. Molecular analyses were used to assess Gli1, hedgehog signalling, and TGFβ1 signalling in the liver and brain of AOM mice and HE patients.

Results: Gli1 expression was increased in brains of AOM mice and in HE patients. Intra-cortical infusion of Gli1 vivo-morpholinos exacerbated the neurologic deficits of AOM mice. Measures to modulate hedgehog signalling had no effect on HE neurological decline. Levels of TGFβ1 increased in the liver and serum of mice following AOM administration. TGFβ neutralizing antibodies slowed neurologic decline following AOM administration without significantly affecting liver damage. TGFβ1 inhibited Gli1 expression via a SMAD3-dependent mechanism. Conversely, inhibiting TGFβ1 increased Gli1 expression.

Conclusions: Cortical activation of Gli1 protects mice from induction of HE. TGFβ1 suppresses Gli1 in neurons via SMAD3 and promotes the neurologic decline. Strategies to activate Gli1 or inhibit TGFβ1 signalling might be developed to treat patients with HE.

Keywords: Sonic hedgehog; Acute liver failure; Azoxymethane; SMAD3; TGFβ1; Hepatic encephalopathy.

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Abbreviations: HE, hepatic encephalopathy; AOM, azoxymethane; TGFβ1, transforming growth factor beta 1; Gli1, glioma-associated oncogene homolog 1; TGFβR2, TGFβ receptor 2; Shh, sonic hedgehog; Ihh, indian hedgehog; HBC, (2-hydropropyl)-β-cyclodextrin; VM, vivo-morpholino; SAG, smoothened agonist; RT-PCR, reverse transcriptase polymerase chain reaction; H&E, hematoxylin and eosin; SIS3, specific inhibitor of SMAD3; CNS, central nervous system; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay.

Introduction

Hepatic encephalopathy (HE) is a neurological complication that can arise following acute or chronic liver damage and is a metabolically-induced, functional disturbance of the brain [1]. The most severe form of HE occurs following acute liver failure, which can be caused by drug-related liver damage, hyperthermic injury, and toxin exposure [2]. The neurological decline observed in HE is caused by toxin accumulation in the blood, which has the ability to cross the blood-brain barrier and generate neurotoxic effects including swelling of astrocytes, cerebral oedema, and dysregulation of water balance [3]. Associated with impairment of astrocyte function is a decrease in neuronal function, which leads to progressive cognitive deficits, motor deficits, and eventually coma [4].

Glioma-associated oncogene homolog 1 (Gli1), a member of the Gli family of transcription factors, is protective in neurological conditions such as ischemic injury, stroke, and Parkinson's disease [5–7]. Activation of Gli1 is a downstream consequence of the hedgehog pathway, via the activation of Smoothened [8]. However, signalling outside of the canonical hedgehog pathway can also regulate Gli activity, such as transforming growth factor beta 1 (TGFβ1) signalling [9,10]. The signalling ligand TGFβ1 binds a receptor complex containing TGFβ receptor 2 (TGFβR2) leading to the phosphorylation/activation of SMAD3, which translocates to the nucleus and regulates transcription [11]. Therefore, understanding both canonical hedgehog signalling and non-canonical pathways, such as TGFβ1, is required to elucidate the regulation of Gli transcription factors during disease states.

Currently, studies addressing the circulating factors released during liver failure and their influence on HE brain pathology are lacking. Recent data suggest that hedgehog signalling is

involved in expansion of liver progenitor cells after injury, and thus, can play a protective role in the liver itself [12]. The hedgehog ligands sonic hedgehog (Shh) and indian hedgehog (Ihh) are released from hepatocytes in various models of liver damage, where they act on liver myofibroblasts and activate endothelial cells [13,14]. Conversely, TGFβ1 signalling has been assessed in chronic liver disease and fibrosis models where it facilitates fibrogenesis while also having anti-inflammatory effects [15]. Necrotic hepatocytes release TGFβ1 into the local microenvironment where they are able to initiate the activation of nearby hepatic stellate cells [16]. Furthermore, studies have found that rats with hepatic failure have elevated levels of serum TGFβ1 [17]. Currently little information exists concerning the roles of either cortical hedgehog or TGFβ1 signalling during HE pathogenesis.

Due to the lack of understanding of neural hedgehog pathway activation during HE, as well as the signalling that occurs between the circulation and brain in this disorder, the current study aims to assess the regulation and effects of neural Gli1 activation during HE and to determine how circulating Shh and TGFβ1 can influence its activation.

Materials and methods

Materials

See Supplementary data section for source of materials.

Murine azoxymethane model of hepatic encephalopathy

Male C57Bl/6 mice (25–30 g; Charles River Laboratories, Wilmington, MA) received a single intraperitoneal injection of 100 μg/g of azoxymethane (AOM) to induce acute liver failure and HE. Following injection, mice were monitored every 2 h (starting at 8 h post AOM) for body temperature, weight and neurological decline. In parallel, mice were pretreated with various agents, aimed at targeting Smoothened, TGFβ1 or Gli1 expression prior to AOM injection. The end point for AOM studies was coma, which was defined as a loss of righting and corneal reflexes (for further methodological details, see Supplementary Materials and methods). All experiments performed complied with the Scott & White Memorial Hospital IACUC regulations on animal experiments (protocol #2012-019-R).

Neuron isolation

Primary neurons were isolated from P1 rat pups and used in molecular analyses. Full details of the isolation and treatments are outlined in the Supplementary Materials and methods.

Human samples

Brain tissue from patients who had HE following liver cirrhosis or aged-matched controls without liver disease were supplied through either the autopsy service from Scott & White Memorial Hospital Department of Pathology (Temple, TX) or the New South Wales Tissue Resource Centre at the University of Sydney. Patient information and cause of death can be found in Table 1.

Table 1. Summary of patient treatment groups.

	Control (n = 7)	Cirrhotic with HE (n = 8)
Male/female ratio	6/1	6/2
Age (avg ± SEM)	51.1 ± 2.51976	59.6 ± 3.93171
Median age (range)	50 (37-61 yr)	58 (40-75 yr)
Cause of death	Cardiac (n = 7)	HE (n = 8)

Liver biochemistry

Plasma alanine aminotransferase and bilirubin levels were assessed using commercially available kits. Alanine aminotransferase measurements were performed using a fluorometric activity assay (Sigma-Aldrich, St. Louis, MO). Total bilirubin was assayed using a total bilirubin ELISA (CusaBio, Wuha, China). All assays and subsequent analyses were performed according to the manufacturer's instructions.

Molecular analyses

Expression and subcellular localization of the described target genes were assessed by real-time PCR (RT-PCR), immunoblotting, immunohistochemistry or immunofluorescence as previously described [18,19]. Phospho-SMAD3 (pSMAD3) and TGFβ1 analyses were performed using an enzyme-linked immunosorbent assay (ELISA) (for specific details, see Supplementary Materials and methods).

Statistical analysis

All statistical analyses were performed using the Graphpad Prism software (Graphpad Software, La Jolla, CA). Results were expressed as mean ± SEM. For data that passed normality tests, the Student *t* test was used when differences between two groups were analysed, and analysis of variance was used when differences between three or more groups were compared followed by the appropriate ad hoc test. If tests for normality failed, two groups were compared with a Mann-Whitney *U* test or a Kruskal-Wallis ranked analysis when more than two groups were analysed. Differences were considered significant when the *p* value was less than 0.05.

Results

Gli1 was activated in the cortex during HE

The AOM model of HE is characterized by a consistent neurological decline towards coma. To better understand disease progression, we performed molecular analyses at times prior to neurological symptom onset, where minor neurological deficits were evident, and at coma (Fig. 1A). In order to validate liver damage, haematoxylin and eosin (H&E) stains and liver biochemistry were assessed at various stages in AOM-treated and vehicle-treated mice. AOM-treated mice displayed progressive liver damage with mice at coma having severe parenchymal damage including diffuse necrosis of hepatocytes and steatosis of the surviving hepatocytes (Fig. 1B). Serum enzyme assays for bilirubin and ALT supported the liver damage assessments (Table 2).

The expression of Gli transcription factors was assessed at various time points after AOM injection. Cortical *Gli1* mRNA was significantly upregulated in AOM mice after development of neurological symptoms and showed greatest elevation at coma (Fig. 1C). This elevation was not observed in the cerebellum of AOM mice (Supplementary Fig. 1A). In addition, there was increased Gli1 immunoreactivity and nuclear translocation in the cortex as an early event after AOM injection that was significantly elevated in later stages of the neurological decline (Fig. 1D). Moreover, Gli1 expression was found to co-localize with the neuronal marker NeuN, but not with the astrocyte marker GFAP, in vehicle and AOM-treated mice at coma, suggesting that Gli1 upregulation occurs primarily in neurons (Fig. 1E). The upregulation of Gli1 was also observed in the cortex of HE patients, with significant increases of Gli1 staining intensity and nuclear translocation compared to normal patients (Fig. 1F). In AOM mice, there were no significant changes in cor-