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Hedgehog regulates cerebellar progenitor cell and medulloblastoma apoptosis

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ABSTRACT

The external granule layer (EGL) is a proliferative region that produces over 90% of the neurons in the cerebellum but can also malignantly transform into a cerebellar tumor called the medulloblastoma (the most common malignant brain tumor in children). Current dogma considers Hedgehog stimulation a potent proliferative signal for EGL neural progenitor cells (NPCs) and medulloblastomas. However, the Hedgehog pathway also acts as a survival signal in the neural tube where it regulates dorsoventral patterning by controlling NPC apoptosis. Here we show that Hedgehog stimulation is also a potent survival signal in the EGL and medulloblastomas that produces a massive apoptotic response within hours of signal loss in mice. This toxicity can be produced by numerous Hedgehog antagonists (vismodegib, cyclopamine, and jervine) and is Bax/Bak dependent but p53 independent. Finally, since glucocorticoids can also induce EGL and medulloblastoma apoptosis, we show that Hedgehog's effects on apoptosis can occur independent of glucocorticoid stimulation. This effect may play a major role in cerebellar development by directing where EGL proliferation occurs thereby morphologically sculpting growth. It may also be a previously unknown major therapeutic effect of Hedgehog antagonists during medulloblastoma therapy. Results are discussed in terms of their implications for both cerebellar development and medulloblastoma treatment.

1. Introduction

The external granule layer (EGL) is a proliferative layer producing over 90% of cerebellar neurons which represent over half of the neurons in the entire brain (the cerebellum actually contains more neurons than the cerebrum) (Andersen et al., 1992; Harvey and Napper, 1988). Once produced, granule neurons migrate past the molecular and Purkinje cell layers before populating the internal granule layer (Fig. 1A). Since Purkinje cells form synaptic connections with granule neurons, neurogenesis is regulated to maintain a consistent ratio between these cell types (Goldowitz and Hamre, 1998). A critical factor regulating EGL proliferation is the Sonic Hedgehog (Hh) pathway which begins with the secretion of Sonic Hh ligand from Purkinje cells onto EGL neural progenitor cells (NPCs) where it binds and inhibits the Patched receptor. Since Patched activation normally represses Smoothened, this diminishes inhibition of Smoothened leading to a downstream activation of Gli transcription factors which are thought to potently increase NPC proliferation (Klein et al., 2001; Wechsler-Reya and Scott, 1999).

There is convincing evidence that EGL NPCs can become tumor-initiating cells that develop into medulloblastomas (MBs), the most common malignant brain tumor in children. For instance, mice with heterozygous mutations in the Patched gene exhibit disrupted inhibition of Smoothened leading to constitutive Hh activation (Goodrich et al., 1997; Oliver et al., 2005). While the EGL normally disappears by two weeks of age, Patched mice exhibit ectopic preneoplastic EGL remnants that are not a MB but have a high probability of progressing into one. Importantly, similar mutations of the Patched gene spontaneously occur in humans with an analogous increase in MB vulnerability (Oliver et al., 2005). Consistent with this finding, up to 30% of human MBs are driven by excessive Hh stimulation (Leary and Olson, 2012). Thus, animal research mirrors clinical findings suggesting that the Hh pathway plays a key role in MB development. This has positioned the Hh pathway as a key area of research for testing MB formation and/or treatment. Much of this interest centers on novel Hedgehog antagonists (HAs) which effectively treat MBs in transgenic mice (Berman et al., 2002;

Abbreviations: HSD2, 11β-hydroxysteroid dehydrogenase Type 2; AC3, activated caspase-3; DEX, dexamethasone; EGL, external granule layer; FA, fluocinolone acetonide; GCs, glucocorticoids; Hh, Hedgehog; HA, Hedgehog antagonist; MBs, medulloblastomas; NPCs, neural progenitor cells.

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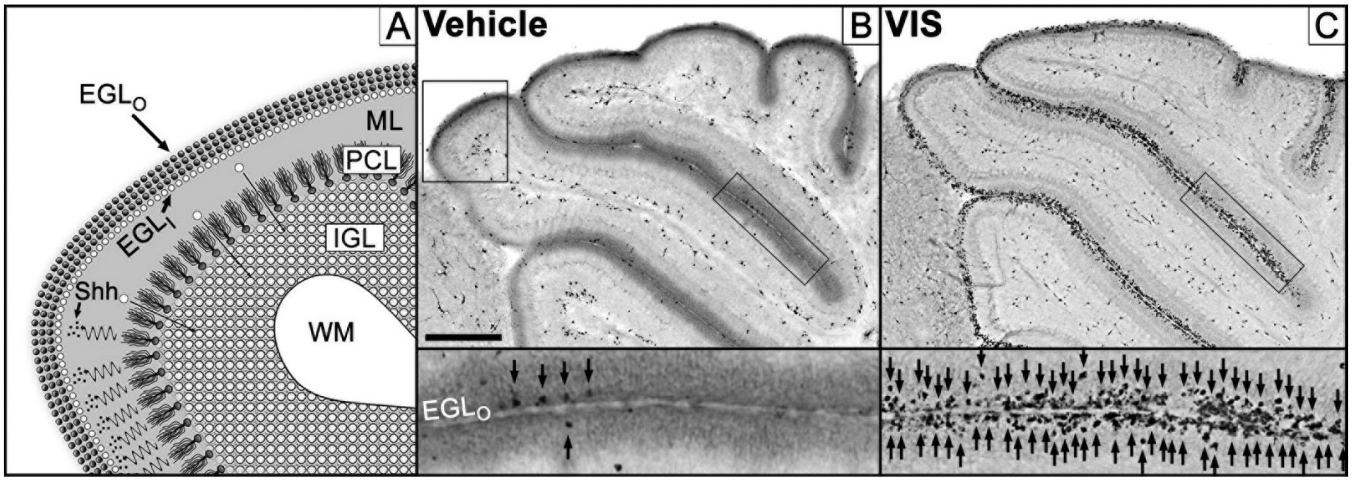

Fig. 1. Hedgehog pathway antagonism produces selective apoptosis in the external granule layer of the developing cerebellum. (A) Magnified diagram of upper left boxed region in (B). The outermost layer of the cerebellum is the transient external granule layer (EGL) composed of an outer germinal matrix populated by neural progenitor cells (EGL_O; dark spheres) and an inner layer (EGL_I; white spheres) where newly formed granule cell neurons congregate and mature. Immature granule cells then migrate (white spheres with arrows) past the molecular (ML) and Purkinje cell layers (PCL) before incorporating into the internal granule layer (IGL) that lies superficial to the cerebellar white matter (WM). Purkinje cells regulate EGL proliferation by secretion of Sonic Hedgehog ligand (Shh; small black dots) that diffuses (squiggly arrows) to the EGL where it stimulates the Hedgehog pathway. (B) Immunolabeling for the apoptotic marker activated caspase-3 (AC3) results in low levels of apoptosis following vehicle treatment while (C) 50 mg/kg vismodegib dramatically increases apoptosis. Insets reflect rectangular black-boxed regions over the EGL with arrows indicating AC3 positive EGL NPCs. Upper left boxed region in (B) diagrammed in (A). Scale Bar: 300 μm. Fig. 1A is a derivative work based on a previously published figure by one of the authors (K.K.N.) under Creative Commons-BY license (Noguchi, 2014).

Romer and Curran, 2005). This enthusiasm recently peaked due to the first Food and Drug Administration approval of the HA vismodegib. While vismodegib is currently approved for treating basal-cell carcinoma, several MB clinical trials have reported anti-tumor effects including some with remarkable results (Gajjar et al., 2010; Robinson, 2013; Rudin et al., 2009).

Interestingly, Hh signaling can have different effects in other regions of the nervous system. Sonic Hh ligand is also produced by the notochord and floor plate in early development which directs dorsoventral patterning of the neural tube by acting as a survival signal (Charrier et al., 2001; Thibert et al., 2003). Thus, NPCs proximal to Hh signaling survive and continue proliferating but more distal cells undergo apoptosis if signaling is lost (Guerrero and Ruiz i Altaba, 2003). Interestingly, blocking NPC apoptosis in the neural tube helps but does not completely rescue the effects of Hh inhibition on the neural tube, suggesting that Hh signaling regulates neural tube development through both apoptosis and proliferation (Guerrero and Ruiz i Altaba, 2003). Based on this research, we tested if Hh stimulation analogously acts as a survival signal in the EGL and MBs. Surprisingly, the loss of Hh signaling produced a massive apoptotic response within a few hours of signal loss.

2. Materials and methods

2.1. Animals and drugs

All procedures were in accordance with the Institutional Animal Care and Use Committee at Washington University in St. Louis and National Institutes of Health guidelines and used mice from both genders. ICR mice (Harlan, Indianapolis, IN, USA) were used in all experiments unless otherwise indicated. p53 knockout (Stock #2101) and Math1-Cre (Stock#11104) mice were purchased from Jackson Laboratories (Bar Harbor, MA, USA). Bax/Bak mice were a kind gift from Scott Oaks (University of California, San Francisco). p53, Math1-Cre, and Bax/Bak mice were maintained on the C57BL/6 strain. Patched mice (Goodrich et al., 1997) were a kind gift from Jane Johnson (UT Southwestern Medical Center) and were on a mixed C57BL/6/129X1/SvJ background. All experiments used mice from both genders.

Since many solvents are neurotoxic in developing brain (Farber et al., 2010; Hanslick et al., 2009; Lau et al., 2012), vismodegib (LC Laboratories, Woburn, MA, USA), cyclopamine (LC Laboratories, Woburn, MA, USA), jervine (Santa Cruz Biotechnology, Dallas, TX, USA), mifepristone (Sigma-Aldrich, St. Louis, MO, USA), itraconazole (Santa Cruz Biotechnology, Dallas, TX, USA), and fluocinolone acetonide (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in palm oil and injected intraperitoneally. Injections of cytosine arabinoside (AraC; Sigma-Aldrich, St. Louis, MO, USA) and dexamethasone sodium phosphate (Voigt Global Distribution LLC, Lawrence, KS, USA) were dissolved in saline. Unless otherwise indicated, animals were perfused 6 hours postinjection for immunolabeling. Following injection, animals were housed separately from their mothers in a veterinary recovery chamber (Mediheat V1200, Dalton, GA, USA) at an ambient temperature of 30°C until perfusion.

2.2. Histology

For immunohistochemistry, animals were deeply anesthetized, transcardially perfused with 4% paraformaldehyde in 0.1 M Tris buffer, and brains sagittally sectioned on a vibratome at 75 μm. Sections were then incubated in a quenching solution (absolute methanol with 3% hydrogen peroxide) for 15 min, a blocking solution (PBS with 2% BSA, 0.2% milk, and 0.1% triton X-100) for an hour, and a primary antibody overnight. Finally, sections were placed in secondary antibody for an hour, reacted with ABC reagents, and incubated in either a chromogen (Vector VIP substrate kit, Vector Laboratories, Burlingame, CA, USA) or fluorescent probes. Photocomposites were taken with a Leica DM400B microscope connected to a Leica DFC310FX camera using Surveyor software V7.0.0.6 MT (Objective Imaging, Cambridge, UK). Immunohistochemistry was performed with antibodies raised against activated caspase-3 (Cell Signaling, Danvers, MA, USA), KI-67 (BD Biosciences, San Jose, CA, USA), or Cre (Millipore, Billerica, MA).

2.3. Semi-quantitative evaluation of activated caspase-3 labeling in the EGL

We have found semi-quantitative evaluation is a highly sensitive measure of EGL apoptosis (Noguchi et al., 2008, 2011). Briefly, a rater blind to treatment examined several midsagittal sections and semi-quantitatively evaluated AC3 in the EGL by assigning a rating to each animal using the following scale as a guide: 0 = no apoptotic profiles