Medulloblastoma Growth Inhibition by Hedgehog Pathway Blockade

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Constitutive Hedgehog (Hh) pathway activity is associated with initiation of neoplasia, but its role in the continued growth of established tumors is unclear. Here, we investigate the therapeutic efficacy of the Hh pathway antagonist cyclopamine in preclinical models of medulloblastoma, the most common malignant brain tumor in children. Cyclopamine treatment of murine medulloblastoma cells blocked proliferation in vitro and induced changes in gene expression consistent with initiation of neuronal differentiation and loss of neuronal stem cell–like character. This compound also caused regression of murine tumor allografts in vivo and induced rapid death of cells from freshly resected human medulloblastomas, but not from other brain tumors, thus establishing a specific role for Hh pathway activity in medulloblastoma growth.

Signaling by the Hh family of secreted proteins was implicated initially in determination of embryonic cell fate, and more recently in maintenance of somatic stem cells and in specification of organ size. The latter role is illustrated in the developing cerebellum, where Hh signaling delays neuronal differentiation and induces proliferation of cerebellar granular neuronal precursors (CGNPs) (1–4). Medulloblastomas, which are aggressive childhood tumors of cerebellar origin, are associated with inappropriate Hh pathway activity (5–8). The activation of this pathway normally requires antagonism of the 12-transmembrane protein Patched (Ptc) by Hh ligand, thus releasing the seven-transmembrane protein Smoothened (Smo) for activation of target genes by the Cubitus interruptus/Gli (Ci/Gli) family of transcription factors (9–11).

Ligand-independent pathway activity in medulloblastoma is caused either by mutations that render Smo insensitive to regulation by Ptc or by mutational inactivation of Ptc. The transcription of Ptc is induced by Hh pathway activity, thus generating a negative feedback loop and serving as a convenient indicator of pathway activation.

We studied Hh pathway activity and function in cerebellar tumors from mice carrying a single mutant allele of the Pch gene (Pch<sup>−/−</sup>) (5). Wild-type Pch mRNA expression was never detected in pure tumor tissue, indicating a lack of functional Pch gene product that should result in Hh pathway activation (see supporting online material, fig. S1). The frequency of medulloblastomas in Pch<sup>−/−</sup> mice was increased by a p53 mutant background (12); loss of p53 function also enabled propagation of these tumors in athymic mice as subcutaneous allografts that displayed diagnostic features of human medulloblastoma (Fig. 1, A and B, and fig. S2) (13). From such allografts independently originating in Pch<sup>−/−</sup> mice, cultured cell lines were derived that lacked p53 function (Fig. 1C), retained a Pch<sup>−/−</sup> genotype (Fig. 1C), and displayed elevated levels of Hh pathway activity (see below). Treatment with the DNA demethylating agent 5-azacytidine (5-azA) restored Pch mRNA expression in each cell line (Fig. 1D) and dramatically reduced activity from the Pch-LacZ reporter (Fig. 1E). As the LacZ gene disrupts the targeted Pch allele, thereby reporting Pch transcription, the observed decrease in β-galactosidase levels indicates a reduction in pathway activity (5, 14). We confirmed the specificity of this effect by stably transfecting cells to express high levels of Gli1, which activates the Hh pathway downstream of Ptc. In such cells, 5-azaC did not block Hh pathway activity (Fig. 1E), despite restoration of Ptc mRNA expression (15). These results indicate that silencing by DNA methylation of the functional Pch allele is the precipitating event in pathway activation and tumor initiation in these animals.

To test the role of Hh pathway activity in tumor growth, we used cyclopamine, a plant-derived pathway antagonist that acts at the level of Smo (14). Medulloblastoma-derived cell lines were cultured with cyclopamine or with tomatidine, another steroidal alkaloid with little effect on the Hh pathway (16). By 72 hours, cyclopamine treatment resulted in a 60 to 80% reduction in growth relative to tomatidine in all tumor-derived cell lines, whereas growth of fibroblast control cells was unaffected (Fig. 2A). Cyclopamine also completely and specifically abolished β-galactosidase activity in all murine medulloblastoma lines (Fig. 2B), demonstrating that the effect of cyclopamine on cell growth parallels the reduction in Hh pathway activity.

The effect of cyclopamine treatment on murine medulloblastoma cell growth is largely mediated by inhibition of cell proliferation, because culture of the PZp53<sup>MED1</sup> allograft line with cyclopamine reduced DNA synthesis by 90%, as compared to tomatidine-treated cells (Fig. 2C), with only low levels of apoptosis (less than 1% of cells) (15). This effect depends on specific pathway inhibition, because PZp53<sup>MED1</sup> cells engineered to overexpress Gli1 retained high levels of β-galactosidase activity (15) and cell growth (fig. S3A) upon treatment with cyclopamine.

To determine the effect of Hh pathway inhibition on cellular pathways that regulate proliferation or differentiation, we examined the expression of cell cycle components and cerebellar neuronal differentiation markers in control and cyclopamine-treated PZp53<sup>MED1</sup> cells. Cyclins D1, D2, E1, and hyperphosphorylated Rb,
which are induced by Shh in CGNP s and promote transit through prereplicative (G1) cell cycle checkpoints (4), are also expressed in tomatidine-treated PZp53MED cells but are suppressed upon treatment with cyclopamine (Fig. 2D, fig S3B). In addition to down-regulation of a proliferative program, cyclopamine treatment appears to initiate differentiation in these cells, as indicated by reduced expression of the neurofilament nestin, a neuronal stem cell marker, and of the bHLH transcription factor Math1, a marker of proliferating CGNP s (17). Furthermore, cyclopamine treatment reduced expression of N-, c-, and L-myc (Fig. 2D, fig S3C) (18) and increased expression of NeuroD, a marker of postmitotic cerebellar granular neurons (Fig. 2D) (19). Morphological neuronal maturation was not observed in vitro, however, indicating that Hh pathway blockade only partially advances differentiation.

We further investigated cyclopamine effects in vivo and found by the 7th day of treatment that subcutaneous injections of the highest cyclopamine dose (1.25 mg/day; ~50 mg/kg) had abolished β-galactosidase activity (Fig. 3A) and growth (Fig. 3B) of medulloblastoma allografts propagated in nude mice. In a second experiment, allograft tumors from Gli-transfected cells (n = 7) grew despite cyclopamine treatment (Fig. 3C), whereas tumors from untransfected cells (n = 4) decreased in volume. Microscopic histologic analysis further demonstrated the complete disappearance of two of these tumors and a dramatic reduction in cell proliferation in the two that remained (fig. S4). These results demonstrate that cyclopamine can induce tumor regression by specific effects on the Hh pathway. No adverse effects were noted in cyclopamine-treated animals.

To explore the potential therapeutic utility of Hh pathway blockade, cells from freshly resected human medulloblastomas were cultured with KAAD-cyclopamine, a potent derivative of cyclopamine (14). Tumor cells initially displayed higher levels of Ptc h mRNA than did normal cerebellum, indicating elevated Hh pathway activity, and this activity was reduced by treatment with either drug (fig. S5, A and B). Treatment with KAAD-cyclopamine also induced a significant decrease in cell viability within 48 hours (Fig. 4A); cell viability continued to decline after prolonged exposure (Fig. 4B), and fewer than 0.1% of cyclopamine-treated cells survived, after 1 week, as compared to typically greater than 10% of vehicle-treated controls. The effects of cyclopamine were similar to those of KAAD-cyclopamine (Fig. 4C) at pathway-inhibitory doses (14). Primary cultures from a glioblastoma and from an ependymoma, in contrast, failed to respond to cyclopamine (Fig. 4A). The dramatic reduction in cell viability of primary cultures from medulloblastomas, but not from other types of brain tumors, and the unaltered growth of embryonic fibroblasts (Fig. 2A) further suggests
that cyclopamine specifically affects medulloblastomas and is not generally cytotoxic.

In contrast to reports suggesting that partial loss of Pch function suffices to initiate tumor formation (20, 21), we found that medulloblastomas arising in Pch−/− mice lack detectable Pch mRNA. The absence of Pch mRNA and consequent pathway activation, apparently caused by DNA methylation of the normal Pch allele, is critical not only for initiation but also for growth of the tumor, because pathway suppression by reactivation of the methylated Pch allele or by cyclopamine treatment blocks growth. Loss of p53 function in murine medulloblastomas appears to favor allograft and cell line growth, perhaps by compromising the ability of cells to undergo apoptosis (22). Human p53 mutations are undetectable in nearly all (92 to 99%) sporadic medulloblastomas (23), which may account for increased apoptosis in primary human medulloblastoma cells cultured with Hh pathway antagonists. Whatever its biological basis, the general requirement for Hh pathway activity in medulloblastoma growth represents a potential therapeutic opportunity, because cyclopamine and other pathway antagonists can be administered in effective doses with no apparent detrimental effects in rodents and other mammals (24).

References and Notes

Fig. 3. Cyclopamine inhibits medulloblastoma growth through specific effects on the Hh pathway. (A) Dose-dependent Pch-LacZ reporter inhibition in PZp53MED1 tumor allografts in tumor tissue from mice treated for 7 days with vehicle (0), or cyclopamine (0.63 or 1.25 mg/day). Error bars represent SEM. (B) Change in tumor allograft volume (%) in the same experiment. In this experiment, treatment was initiated after tumors reached an average size of 181 mm³. (C) Cyclopamine treatment induces regression of control-transfected tumors but not of tumors overexpressing the downstream Hh effector Gli1. Athermic mice bearing tumor allografts from control or Gli1-transfected PZp53MED1 cells were grown to ≈125 mm³ before treatment with daily subcutaneous injections of cyclopamine (1.25 mg/day) for 24 days.

Fig. 4. Cyclopamine causes loss of viability of cultured tumor cells from primary human medulloblastomas. (A) Loss of cell viability in response to KAADD-cyclopamine (1 μM) 48 hours after surgical resection of tumor. Viability was assessed by absence of propidium iodide and annexin V-fluorescein isothiocyanate staining. Error bars represent standard error of the mean. (B) Loss of cell viability in response to KAADD-cyclopamine (1 μM) over time. (C) Dose response curves measuring loss of cell viability in response to increasing concentrations of cyclopamine (0 to 3 μM) and KAADD-cyclopamine (0 to 1 μM) in two medulloblastomas.