Notch1 and Notch2 Have Opposite Effects on Embryonal Brain Tumor Growth

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ABSTRACT

The role of Notch signaling in tumorigenesis can vary; Notch1 acts as an oncoprotein in some neoplasms, and a tumor suppressor in others. Here, we show that different Notch receptors can have opposite effects in a single tumor type. Expression of truncated, constitutively active Notch1 or Notch2 in embryonic brain tumor cell lines caused antagonistic effects on tumor growth. Cell proliferation, soft agar colony formation, and xenograft growth were all promoted by Notch2 and inhibited by Notch1. We also found that Notch2 receptor transcripts are highly expressed in progenitor cell-derived brain tumors such as medulloblastomas, whereas Notch1 is scarce or undetectable. This parallels normal cerebellar development, during which Notch2 is predominantly expressed in proliferating progenitors and Notch1 in postmitotic differentiating cells. Given the oncogenic effects of Notch2, we analyzed its gene dosage in 40 embryonal brain tumors, detecting an increased copy number in 15% of cases. Notch2 gene amplification was confirmed by fluorescence in situ hybridization in one case with extremely high Notch2 mRNA levels. In addition, expression of the Notch pathway target gene Hes1 in medulloblastomas was associated with significantly shorter patient survival (P = 0.01). Finally, pharmacological inhibition of Notch signaling suppresses growth of medulloblastoma cells. Our data indicate that Notch1 and Notch2 can have opposite effects on the growth of a single tumor type, and show that Notch2 can be overexpressed after gene amplification in human tumors.

INTRODUCTION

Embryonal brain tumors, known as medulloblastomas in the cerebellum and primitive neuroectodermal tumors (PNETs) elsewhere in the central nervous system (CNS), are among the most frequent causes of cancer death in children (1). Because of their primitive appearance and potential for divergent differentiation, these malignant lesions have long been thought to arise from progenitor cells in germinal epithelia situated periventricularly and on the cerebellar surface (2, 3). Neural stem-like cells have recently been isolated from human medulloblastoma (4, 5), which supports this proposed histogenesis and which suggests that pathways affecting CNS progenitors might play a role in embryonal brain tumor pathology.

The Wnt (6), Hedgehog (7, 8), and Notch (9–11) developmental signaling cascades all regulate CNS stem cell dynamics, and both the Wnt and Hedgehog pathways are mutually activated in a subset of medulloblastomas (1, 12). The role played by Hedgehog signaling is particularly significant, with aberrant activation of this pathway driving proliferation of cerebellar granule cell precursors in vitro and causing tumors in transgenic mice (8, 13). In contrast to Hedgehog and Wnt, the role of Notch signaling in medulloblastoma pathogenesis is unknown. However, in situ hybridization (14, 15), murine reporter knockin (16), and reverse transcription-PCR (RT-PCR; ref. 15) analyses have all shown that Notch2 is expressed in rodent cerebellar granule cell precursors. In addition, activated Notch2 acts as a mitogen for cerebellar granule cell precursors (15). Taken together, these data suggest Notch2 signaling may be involved in cerebellar neoplasia. Interestingly, Notch1 is not expressed in proliferating cerebellar precursors (14, 15) but is, instead, found in differentiated internal granule layer neurons (17).

Although Notch was initially described as a gene regulating epidermal and neuronal cell fate decisions in Drosophila (18), over time it has become clear that the effects of Notch signaling are dependent on cellular context (19). This is particularly true for vertebrates, in which the presence of four Notch receptors and multiple ligands in the Jagged and Delta families leads to a complex array of possible receptor-ligand combinations (20–22). Signaling is initiated when ligands bind a Notch receptor and permit the γ-secretase–mediated proteolytic release of the Notch intracellular domain (NICTD). NICTD then translocates into the nucleus, in which it interacts with the transcriptional cofactor CBFI and transactivates gene targets such as those in the Hes and Hey families (22).

Notch signaling has been implicated in several neoplasms arising outside the CNS. Dysregulated expression of Notch receptors or other pathway components has been demonstrated in both hematopoietic tumors and in cervical, pancreatic, and colon carcinomas (19, 21, 23). In human T-cell lymphoblastic leukemias, Notch1 can be activated by 9;7 chromosomal translocations resulting in expression of a truncated, constitutively active receptor (24). Bone marrow cells transduced with activated Notch1 cause similar leukemias when transplanted into lethally irradiated mice (25). Another pathway member, MAML2, is activated by translocations in mucoepidermoid carcinoma (26). Whereas genetic alterations activating Notch2 have not yet been identified in human neoplasms, truncated forms of the Notch2 receptor induce thymic lymphomas in cats (27). Notch2 can also transform rat kidney cells in vitro in combination with E1A (28). Given this oncogenic potential, and the mitogenic effects of Notch2 in cerebellar granule cells progenitors, we investigated whether Notch2 signaling regulates the growth of medulloblastomas and other CNS embryonal tumors, hypothesizing it would act as an oncoprotein.

MATERIALS AND METHODS

Clinical Material and Immunohistochemistry. 3,3’-diaminobenzidine (DAB)-immunoperoxidase staining was performed with the DAKO Vectastain Elite system with Notch2 (M20; 1:200) from Santa Cruz Biotechnology (Santa Cruz, CA) Hes1 (1:3,000) from Dr. Teseu Sudo (Toray Industries, Kanagawa, Japan), and Ki67 (1:1,000) from DAKO (Carpinteria, CA). Peptide competition eliminated immunoreactivity for Notch2. Medulloblastoma/PNET surgical specimens were from The Johns Hopkins Hospital Department of Pathology. Frozen tissue from normal fetal cerebella was obtained from the Brain and Tissue Bank for Developmental Disorders (University of Maryland, Baltimore). The medulloblastoma tissue array was constructed as described previously (29). These studies were approved by the Internal Review Board of the Johns Hopkins University School of Medicine. Immunohistochemistry on DAOY cells was performed with standard techniques. In brief, cells were seeded in 6-well tissue culture plate and were allowed to adhere overnight.
After infection with AdN1C2 or AdDfTggal for 48 hours, cells were fixed with 4% paraformaldehyde, permeabilized with 0.4% Triton X-100, blocked in PBS/5% bovine serum albumin, then incubated with anti-Notch2 antibody [C651.6DbHN originally developed by Dr. Artavanis-Tsakonas (30) and obtained from the University of Iowa Developmental Hybridoma Studies Bank, 1:10 dilution] for 2 hours. Cy3-conjugated secondary antibody incubations and 4',6-diamidino-2-phenylindole (DAPI) counterstains were performed with appropriate wash steps before mounting and visualization with a fluorescence microscope (Zeiss, Germany).

Polymerase Chain Reaction. Quantitative RT-PCR was performed as described previously (31), with all reactions normalized to actin (Applied Biosystems, Foster City, CA). The primers and probes for Notch1, Notch2, and Hes1 are: Notch1 forward (F) 5'-CCAGCTTGGTCTCTCTGA3'- Notch1 reverse (R) 5'-AAGCAAGATATGGAGACAGAGAACAAGACGAGGAGAGA3'- Notch2 F 5'-GGGCTAAATCGCTCTGAGCTT-3' Notch2 R 5'-GGAGGACACATCTCACTATGTCA-3', Notch2 probe 5'-CAGCCGCTTCACAGGGAGAGAACA3'- Hes1F 5'-CGTTCTAGCTACCTGCTCAGA3'- Hes1R 5'-GTCGGTGGTGGTTGCTGGA3'- Hes1 probe 5'-CTGGCTGTGAGACAGCAACAGGACGGTGT3'.

RESULTS

Differential Expression of Notch1 and Notch2 in Normal and Neoplastic Cerebellar Cells. We first sought to define the expression pattern of Notch transcripts in embryonal brain tumors derived from the external germinal layer (EGL) and ventricular zone, predicting lower expression of Notch1 than of Notch2 in cerebellar tumors. To obtain quantitative data on gene expression, we used real-time RT-PCR to measure Notch1 and Notch2 levels in mRNA extracted from snap-frozen brain tumors. We compared Notch expression in tumors that in rapidly proliferating fetal cerebellum in which the pathway should be active, based on developmental studies in rodents. Notch2 mRNA was present in all 30 medulloblastomas examined, and 25 expressed higher levels than did fetal cerebellum (Fig. 1A). In parallel with its absence from the proliferating outer EGL in normal development, Notch1 was undetectable in 9 of 22 medulloblastomas, and only 3 of the 13 positive cases had expression equal to or higher than that in fetal cerebellum. Hes1 message was also detected in all cases, although only five had levels higher than fetal cerebellum (data not shown). In medulloblastoma, Notch2 and Hes1 expression (Fig. 1B) showed a significant positive correlation ($P = 0.04$), whereas Notch1 and Hes1 did not, which suggests that Notch2 may regulate Hes1.

Fluorescence In situ Hybridization. Fluorescence in situ hybridization (FISH) with probes generated by using bacterial artificial chromosomes (BACs) CTD-2574B15 (Notch2), and 260I23 (1p32 reference probe) was performed as described previously (32). The cytogenetic localization of the Notch2 BAC clone (1p11–13) was confirmed on metaphase preparations.

Cell Culture and Xenograft Experiments. DAOY and PFSK cell lines were obtained from the American Type Culture Collection and maintained in Richter’s zinc option media supplemented with 10% fetal bovine serum unless otherwise noted; UW228 cells were the kind gift of Dr. John Silber (Department of Neurological Surgery, University of Washington, Seattle, WA) and were grown in DMEM/F-12 with 10% fetal bovine serum. The Notch intracellular domain (NICD) adenosinovirus construction and procedures for infection have been described previously (33). Stable transfectants were established by selection with G418 with standard techniques. Flow cytometric analysis of cell death was performed in medulloblastoma (DAOY, UW228) and PNET (PFSK) cell lines. In F, the goat polyclonal Notch2 antibody used in our immunohistochemical studies identified the same M$\alpha$ 120,000 transmembrane Notch2 band in DAOY protein extracts as the anti-Notch2 monoclonal C651. An appropriately sized M$\alpha$ 31,000 Hes1 band was also identified in these extracts.
expression in EGL-derived tumors. We extended our analysis to 12 supratentorial PNETs. Interestingly, these extracerebellar lesions had significantly higher levels of Notch1 (Fig. 1C) and Hes1 mRNA (data not shown) than did medulloblastoma, which supported an earlier study showing that these microscopically similar tumors can have different gene expression profiles (35). Although Notch2 expression was not statistically different in medulloblastomas and PNETs, it was higher in the latter tumors, including one PNET with Notch2 mRNA levels >20,000-fold higher than in fetal cerebellum (Fig. 1D). The Notch receptors and Hes1 were also expressed in two medulloblastoma cell lines (DAOY, UW228) and in the supratentorial PNET cell line PFSK (Fig. 1E). Notch pathway proteins were also detected in these cells. The $M_r \sim 120,000$ transmembrane Notch2 receptor was detected in DAOY protein extracts by using either polyclonal antiserum or a previously described (30) Notch2-specific monoclonal antibody (Fig. 1F). The $M_r$ 31,000 Hes-1 protein was also abundant in DAOY cells. Consistent with its low mRNA level, the transmembrane Notch1 protein band was only faintly detected in DAOY cells by using a monoclonal antibody (data not shown), and was undetectable with the polyclonal antibody unless overexpressed (Fig. 3B).

**Activation of Notch Signaling in Medulloblastomas Is Associated with Worse Clinical Outcomes.** Because Notch2 signaling is linked to proliferation, and highly proliferative tumors are more aggressive clinically, we investigated the relationship between Notch pathway activity and patient survival. Of the 41 medulloblastoma and PNET patients with Notch2 mRNA expression data and clinical follow-up available, only 14% with below-median Notch2 levels died as compared with 45% of those with above-median Notch2 expression. However, these survival differences were not statistically significant, perhaps because of the limited follow-up available for these relatively recent cases in which frozen material was collected. We, therefore, also analyzed Notch pathway activity immunohistochemically by using a previously described medulloblastoma tissue array (29) containing paraffin-embedded specimens from patients with longer follow-up times. Expression of the Notch target Hes1, which, as described above, is associated with Notch2 activity, was graded semiquantitatively in 35 medulloblastomas remaining on the array (Fig. 2A). Notch2 could not be analyzed on the array because the tissue block was depleted by earlier analyses. The 11 patients with Hes1 immunopositive tumors (+ to ++++) had a higher mortality rate (64%) than the 24 with Hes1-immunonegative tumors (25%). Log rank analysis of Kaplan–Meier survival curves confirmed the statistical significance ($P = 0.01$) of the shorter survival in patients with Hes1-immunopositive tumors (Fig. 2B). In addition, of the 11 positive cases, the 4 tumors with more intense Hes1 staining (+++ or +++++) all came from patients who did not survive. Thus, components of the Notch pathway involved in normal development are expressed in embryonal brain tumors, and expression of the Notch pathway target Hes1 is prognostic of worse clinical outcomes.

**Increased Notch2 Gene Dosage in Central Nervous System Embryonal Tumors.** We were curious about the mechanisms by which Notch signaling might be activated in medulloblastoma/PNET. In some embryonal brain tumors, amplification of oncogenes can greatly increase their mRNA levels (31). Because of the extremely high level of Notch2 transcript in several PNET cases, we examined Notch2 gene dosage to determine whether chromosomal alterations might account for this overexpression. Differential PCR analysis comparing three separate regions of the Notch 2 gene to a STS reference probe on 1q revealed that 6 of 40 (15%) medulloblastoma/PNET had increased Notch2 gene copy number. FISH analysis of the Notch2 locus was successful in three of these six cases and confirmed increased gene dosage. One PNET contained >12 copies of Notch2 in ~10% of neoplastic cells (Fig. 2C). No amplification of a 1p32...
reference probe was seen in this case, which indicated that the ampiclon encompassed only a fraction of the 1p chromosomal arm. Gene amplification in this tumor was associated with a Notch2 mRNA level 20,000-fold higher than in fetal cerebellum (arrow, Fig. 1D), as well as with high Notch2 (Fig. 2C) and Hes1 protein levels, strongly suggesting that the chromosomal alteration affected Notch signaling. In the other two tumors successfully analyzed by FISH, all or most of chromosomal arm 1p was gained, with 4 to 16 copies of both the 1p11–13 and 1p32 sequences present in the majority of tumor cells (data not shown). Therefore, although there was not evidence of Notch2 amplification in these two cases in comparison with the 1p control, there was nevertheless a significant gain in copy numbers.

**Notch1 and Notch2 Have Opposite Effects on the Growth of Medulloblastoma/PNET Cells.** To test whether Notch1 and Notch2 activity alters the growth of embryonal tumors in vitro, we expressed truncated, constitutively active forms of the receptors in established human medulloblastoma and PNET cell lines. Infection of DAOY cells with adenovirus encoding the intracellular domain of Notch2 (NICD2) resulted in abundant nuclear Notch2 protein in >50% of tumor cells (Fig. 2D). Uninfected DAOY cells contained lower levels of Notch2 protein, and it was less commonly present in the nucleus. Infection with the NICD2 adenoviral constructs also led to a 3-fold or greater increase in respective mRNA levels after 48 hours (Fig. 3A), appearance of a truncated Notch2 band on Western blots (Fig. 3B), and induction of Notch pathway targets (Fig. 3A and Fig. 5A). Consistent with the low Notch1 mRNA level in DAOY cells, Notch1 protein was detected in these cells only after infection with AdNICD1 (Fig. 3B).

Interestingly, activation of the two Notch receptors revealed opposing effects on proliferation. Using fluorescent activated cell sorting to analyze tumor cell cycle changes, we found that NICD1 inhibited proliferation by 47%, whereas NICD2 stimulated proliferation by 47%, whereas NICD2 stimulated proliferation by 27%, as determined by the percentage of DAOY cells in S phase (Fig. 3C). The G1-G0 fraction rose from 37% to 70% in AdNICD1-infected DAOY cells compared with AdβGal- infected cells, consistent with a G1 arrest of the cell cycle. NICD2 expression was accompanied by a decrease in G1-G0 fraction. These experiments were repeated four times with comparable results. Similar cell cycle changes in two additional embryonal brain tumor lines (PFSK, UW228) suggest that the antithetical proliferative effects are a general property of Notch in at least a subset of embryonal tumors. The disparate effects of Notch1 and Notch2 in embryonal tumors also extended to in vitro assays of tumorigenicity, with NICD1 inhibiting, and NICD2 stimulating, clone formation in soft agar several-fold (Fig. 3D).

Suppression of Notch1 or Notch2 expression by RNA interference resulted in changes opposite those seen in NICD1- and NICD2-expressing cells. Transfection of siRNA-targeting Notch2 resulted in a 90% or greater knockdown of Notch2 mRNA (Fig. 4A) or protein (Fig. 4B) in DAOY cells. Notch2 siRNA also reduced levels of three Notch target genes, evidence of functional inhibition of Notch2 signaling (Fig. 4C). Notch1 siRNA effectively reduced Notch1 mRNA levels after transfection into PFSK cells (Fig. 4A), but protein level reductions were less pronounced (Fig. 4B). Finally, although Notch1 knockdown promoted cell proliferation, reduction of Notch2 expression decreased the fraction of cells in S phase (Fig. 4D). These loss-of-function studies strongly suggest that the inhibitory effects of NICD1 are not the result of nonspecific toxicity, and that opposing activities of Notch1 and Notch2 stem from unique functions of each receptor in these embryonal brain tumor cells.

To facilitate longer-term assays, we constructed DAOY cell lines stably transfected with the intracellular domains of Notch1 (D: NICD1) or Notch2 (D-NICD2). Growth alterations in these cells paralleled those seen after NICD delivery by adenoviral infection,
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Fig. 4. Effects of Notch siRNA knockdown and stable NICD expression on proliferation and growth. In A, quantitative RT-PCR analysis showed that siRNAs, targeting Notch1 or Notch2, reduced their respective mRNA levels by 90% or more, 48 hours after transfection into PFSK or DAOY cells. In B, Notch2 protein expression also decreased >80% in DAOY cells 48 hours after siRNA treatment. Notch1 protein levels in PFSK cells decreased less significantly after Notch1 siRNA treatment (siN1) compared with control siRNA (siC). In C, Notch2 knockdown was accompanied by decreased mRNA levels in several Notch targets. In D, loss of Notch1 (N1 siRNA) and Notch2 (N2 siRNA) caused effects on DAOY cells proliferation that were the opposite of the effects seen after their activation. In E, stably transduced DAOY clones, expressing truncated Notch1 (D:NICD1) or Notch2 (D:NICD2), had disparate long-term growth rates in low serum (0.5% fetal bovine serum) as measured by MTS assay. In F, D:NICD2- and D:NICD1-flanked xenografts in nude mice also displayed significant differences in weight when removed after 7 weeks (n = 8 per group). In G, the frequency of mitotic figures was 2-fold lower in NICD1-expressing cells compared with untransfected DAOY cells. In H, Ki67 immunostains highlight the higher proliferation rates associated with Notch2 activity.

with a representative D:NICD1 subclone growing 2.8-fold slower and a D:NICD2 subclone growing 2-fold faster than the progenitor cell line (Fig. 4E). Injecting these cells subcutaneously in nude mice provided additional evidence for the growth-suppressing activity of Notch1, with significantly decreased xenograft weights after 7 weeks (Fig. 4F). Mitotic cell counts confirmed a statistically significant decrease in the proliferation of D:NICD1 xenografts compared with D:NICD2 (P < 0.001; Fig. 4G). Microscopic examination of D:NICD1 xenografts revealed mainly sparse tumor cells admixed with residual Matrigel, and even the most cellular regions of these lesions had low proliferation indices measured by Ki67 staining (Fig. 4H). In contrast, D:NICD2 xenografts were densely cellular with abundant Ki67 positive cells. Immunohistochemical staining of xenografts with antibodies specific for cleaved caspase 3 revealed no significant differences in apoptosis between NICD1- and NICD2-expressing cells (data not shown).

**Notch1 and Notch2 Have Different Effects on the Expression of Hes1 and Hedgehog Pathway Components.** The canonical Notch signaling pathway regulates expression of downstream basic helix-loophelix (bHLH) transcription factors, including, among others, Hes1 and Hey1 (22). We wondered whether such factors might be differentially regulated by Notch1 and Notch2 in embryonal tumors. Interestingly, although Hes1 and Hey1 were both up-regulated in NICD2-expressing DAOY cells, NICD1 activity was associated with increased expression of only Hey1 (Fig. 5A). Similar expression changes were seen in PFSK cells (data not shown). Thus, differential activation of direct Notch targets may account for some of the opposing tumorigenic effects we observe. The Hedgehog pathway is a second possible mediator of the disparate effects of Notch on medulloblastoma growth, because it is known to regulate medulloblastoma proliferation, and suppression of Hedgehog signaling by Notch1 was recently reported in the skin (36). We indeed found profound suppression of three targets of the Hedgehog pathway (PTCH, Gli1, and Gli2) after adenovirus-mediated delivery of NICD1 (Fig. 5B). In contrast, AdNICD2 infection increased expression of these genes. Similar results were seen in DAOY cells stably transfected with NICD1 and NICD2. Thus, although the Notch1 and Notch2 receptors are structurally similar, their activation can induce opposing transcriptional programs. Loss of Notch receptor expression also affected Hedgehog activity, but siRNAs for both Notch1 and Notch2 increased expression of Hedgehog pathway targets, suggesting that endogenous Notch activity in DAOY cells may act mainly to suppress Hedgehog (Fig. 5C).

Because Hes1 was significantly associated with poor clinical outcomes in embryonal brain tumor patients and was also positively regulated by Notch2, we examined the ongoing requirement for Hes1 in the growth of medulloblastoma/PNET cells. siRNA sequences targeting Hes1 reduced its mRNA levels by >60% in DAOY and PFSK cell lines 48 hours after transfection. Decreased Hes1 expression was accompanied by a significant slowing in DAOY and PFSK cell growth (Fig. 5E). Thus, Hes1 may mediate some or all of the growth-promoting properties of Notch2.

**Pharmacological Inhibition of Notch Signaling Arrests Medulloblastoma Growth.** Current therapies for embryonal brain tumors cure only ~60% of patients and are associated with significant long-term side effects (1). Our RNA interference data indicate an ongoing requirement for Notch2 activity for medulloblastoma growth and suggest that the inhibition of Notch signaling might significantly reduce the growth of embryonal brain tumors in which Notch2 activity predominates over that of Notch1. γ-secretase inhibitors prevent Notch activation by blocking the cleavage event releasing the active intracellular domain (37, 38). One such compound, DFK-167, reduced Hes1 expression in DAOY cells in a dose-dependent fashion (Fig. 5F). The apparent IC50 was similar to the 20- to 30-μmol/L value reported in Chinese hamster ovarian cells (39), and cell growth was inhibited 82% by a 50-μmol/L concentration of the compound (Fig. 5F). Importantly, growth of D:NICD2 cells, which express already truncated receptor and do not require γ-secretase for Notch2 activity, was not inhibited by 50 μmol/L DFK-167, which indicated that DFK-167 suppresses growth through specific effects on Notch sig-
Notch1 in postmitotic cells rather than in proliferating progenitors, suggesting it may promote cell cycle exit and neuronal differentiation in this developmental context.

The effects of Notch1 and Notch2 on medulloblastoma growth recapitulate these apparent developmental differences. We demonstrate that Notch1 activity inhibits proliferation of medulloblastoma cells, whereas Notch2 promotes their growth \textit{in vitro}. Notch1 was initially thought to be oncogenic, but is now known to suppress tumor formation in some contexts. The Notch1 intracellular domain can inhibit the proliferation of small-cell lung cancer (41), and down-regulation of Notch1 expression is likely involved in the progression of some cervical cancers (42). Nicolas et al. (36) have recently inactivated Notch1 in murine epidermis and have shown that it acts as a tumor suppressor gene in the skin. Although these earlier studies highlight the potential of Notch1 to act as an oncogene or tumor suppressor gene in different neoplasms, we demonstrate for the first time that Notch1 and Notch2 can have antagonistic affects on the growth of a single tumor type.

This suggests that a deeper understanding of the various Notch receptors and their downstream targets will be required for effective clinical interventions. Interestingly, in skin, as in the developing cerebellum, Hedgehog activity plays a key role in the proliferation of progenitor cells, and regulation of Hedgehog signaling seems to be one mechanism by which Notch1 suppresses tumor formation in keratinocytes (36). We demonstrate that, in cerebellar tumor cells, Notch1 activity suppresses expression of Hedgehog targets, whereas Notch2 activation up-regulates the same genes. Notch1 and Notch2 also have opposing effects on the expression of Hes1, a direct effector of Notch signaling, which suggests that differential regulation of both direct and indirect Notch targets may underlie the opposing growth phenotypes that we observe.

The expression profiles of Notch1 and Notch2 in primary medulloblastomas are consistent with their opposite effects on tumor growth \textit{in vitro}. Using quantitative RT-PCR, we found that Notch1 transcripts were undetectable or scarce in 22 medulloblastoma specimens, whereas Notch2 levels were generally higher than those in rapidly growing fetal cerebellum. Colleagues in another laboratory have found Notch1 mRNA levels to be somewhat higher in medulloblastomas than we did. The cause of this discrepancy is not clear, but the alterations in mRNA level that we detect after Notch overexpression or siRNA knockdown suggest that our primers and probes are measuring the appropriate transcripts.

Notch2 expression correlated with that of Hes1, a well-characterized pathway target. Significantly, expression of Hes1 protein in medulloblastoma specimens was associated with worse clinical outcomes, directly implicating the pathway in the pathobiology of primary human lesions. Additional evidence for the role of Notch2 in human tumors comes from our analysis of gene dosage. Notch2 gene copy number was increased in 15% of the 40 embryonal tumor specimens examined, and in one PNET, amplification of the Notch2 locus was associated with mRNA levels 20,000-fold higher than those in fetal cerebellum. Gene amplification, thus, seems to cause Notch2 overexpression in a small subset of human brain tumors. The mechanisms for increased Notch2 expression in tumors without chromosomal alterations, and whether signaling in these tumors is ligand dependent, remain to be determined.

In summary, we demonstrate that, in embryonal brain tumors, Notch1 and Notch2 have opposing effects on growth that parallel their functions in normal development. These strikingly antagonistic changes are highly unusual, and further characterization of the imme-

\textbf{DISCUSSION}

In this study, we demonstrate that the Notch signaling pathway plays a significant role in the growth of embryonal brain tumors such as cerebellar medulloblastoma. Notch activity has been previously shown to regulate the granule cell progenitors from which medulloblastomas form. Solecki et al. (15) reported that Notch2 and Hes1 promote the proliferation of murine cerebellar granule cell progenitors \textit{in vitro} and in cerebellar slice cultures. Interestingly, they detected
diate effectors and downstream targets of Notch1 and Notch2 in these tumors should provide insight into the growth control functions of the Notch pathway. Finally, using RNA interference and inhibition of γ-secretase, we document an ongoing requirement for Notch2 and Hes1 in medulloblastoma growth, suggesting that modulation of Notch signaling may be therapeutically useful in one of the most common malignant solid tumors of childhood.

ACKNOWLEDGMENTS

We thank A. Chaudhry, L. Khaki, S. Karhadkar, and R. Abounader for technical assistance and advice; L. Miele for providing siRNA sequences for Notch1; and T. Sudo for Hes1 antibody. The C651 monoclonal antibody developed by Spyros Artavanis-Tsakonas was obtained from the University of Iowa Developmental Hybridoma Studies Bank, under the auspices of the National Institute of Child Health and Human Development.

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Cancer Res 2004;64:7787-7793.

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