Notch Pathway Inhibition Depletes Stem-like Cells and Blocks Engraftment in Embryonal Brain Tumors

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Abstract

The Notch signaling pathway is required in both nonneoplastic neural stem cells and embryonal brain tumors, such as medulloblastoma, which are derived from such cells. We investigated the effects of Notch pathway inhibition on medulloblastoma growth using pharmacologic inhibitors of γ-secretase. Notch blockade suppressed expression of the pathway target Hes1 and caused cell cycle exit, apoptosis, and differentiation in medulloblastoma cell lines. Interestingly, viable populations of better-differentiated cells continued to grow when Notch activation was inhibited but were unable to efficiently form soft-agar colonies or tumor xenografts, suggesting that a cell fraction required for tumor propagation had been depleted. It has recently been hypothesized that a small population of stem-like cells within brain tumors is required for the long-term propagation of neoplastic growth and that CD133 expression and Hoechst dye exclusion (side population) can be used to prospectively identify such tumor-forming cells. We found that Notch blockade reduced the CD133-positive cell fraction almost 5-fold and totally abolished the side population, suggesting that the loss of tumor-forming capacity could be due to the depletion of stem-like cells. Notch signaling levels were higher in the stem-like cell fraction, providing a potential mechanism for their increased sensitivity to inhibition of this pathway. We also observed that apoptotic rates following Notch blockade were almost 10-fold higher in primitive nestin-positive cells as compared with nestin-negative ones. Stem-like cells in brain tumors thus seem to be selectively vulnerable to agents inhibiting the Notch pathway. (Cancer Res 2006; 66(15): 7445-52)

Introduction

Medulloblastoma and other embryonal brain tumors are thought to arise primarily from neural stem/precursor cells of the ventricular zone and cerebellar external germinal layer (1–3). Pathways such as Wingless, Hedgehog, and Notch, which control the specification, proliferation, and survival of nonneoplastic neural precursors, are also all aberrantly activated in such tumors, suggesting a molecular link between neural stem cells and medulloblastoma (4–9). These developmentally significant signaling pathways may be required for the long-term propagation of neoplastic growth and that CD133 expression and Hoechst dye exclusion (side population) can be used to prospectively identify such tumor-forming cells. We found that Notch blockade reduced the CD133-positive cell fraction almost 5-fold and totally abolished the side population, suggesting that the loss of tumor-forming capacity could be due to the depletion of stem-like cells. Notch signaling levels were higher in the stem-like cell fraction, providing a potential mechanism for their increased sensitivity to inhibition of this pathway. We also observed that apoptotic rates following Notch blockade were almost 10-fold higher in primitive nestin-positive cells as compared with nestin-negative ones. Stem-like cells in brain tumors thus seem to be selectively vulnerable to agents inhibiting the Notch pathway.

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Materials and Methods

Cell culture. The DAOY, PFSK, D283Med, and D425Med cell lines were obtained from the American Type Culture Collection and maintained in MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) unless otherwise noted. Cell pools and stable subclones transfected with Notch2 intracellular domain (NICD2) were generated as previously described, and, unless otherwise noted, the DAOY:NICD2 subclone used is the same as the one previously reported (8). The low-passage medulloblastoma line MB2 was derived from a tumor resected at Johns Hopkins Hospital and analyzed at passage 8 to 9. It was finely minced, triturated, and maintained in MEM with 10% FBS. For treatment studies, cells were plated and allowed to grow overnight in medium containing 10% FBS; then medium was replaced the next morning with low-serum (0.5% FBS) MEM containing γ-secretase inhibitor dissolved in DMSO at the concentrations indicated. RNA and protein extractions and all cell-based assays were done 48 hours after drug application unless otherwise noted. All experiments with error bars were done in triplicate and shown as mean values with SE unless otherwise noted. Cell mass was measured using CellTiter assays according to the instructions of the manufacturer (Promega, Madison, WI). Cell number and viability were assessed using the Guava PCA and Viacount reagent according to instructions (Guava Technologies, Heyward, CA). Soft agar assays were done as previously described, with colonies counted using an automated reader (8).

γ-Secretase inhibitor synthesis. The potent γ-secretase inhibitor [11-endo]-X-(5,6,7,8,9,10-hexahydrop-6,9-methanobenzo[a]j[8]annulen-11-yl)-thiophene-2-sulfonamide was listed as compound 18 in the recent report by Lewis et al. (39) describing its synthesis and testing, and we refer to it as GSI-18. It was synthesized as previously described (39) and its identity and quality were confirmed by nuclear magnetic resonance and mass spectral analysis. The biological activity of this inhibitor against γ-secretase was confirmed using in vitro and cell-based assays as previously described (40).

Immunocytochemistry. The cell lines DAOY and MB2 were seeded in 12-well tissue culture plates and allowed to adhere overnight. After treating with 2 μM/L GSI-18 for 48 hours, cells were washed with PBS and fixed using 4% paraformaldehyde solution in PBS at room temperature for 30 minutes. Cells were then permeabilized with 0.4% Triton X-100 in PBS for 5 minutes at room temperature, washed with PBS, and incubated in 5% bovine serum albumin (BSA)-PBS for 1 hour and then in 1:1,000 anti-nestin antibody (Chemicon, Temecula, CA) or 1:500 anti–cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) in 1% BSA-PBS for 2 hours. After washing, cells were permeabilized with 0.4% Triton X-100 in PBS for 30 minutes. Cells were then incubated with 1:100 anti-nestin antibody (Chemicon) or 1:1,000 anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) in 1% BSA-PBS for 2 hours. After washing, cells were permeabilized with 0.4% Triton X-100 in PBS for 30 minutes. Nuclear membranes were stained with DAPI (Invitrogen) and cell nuclei were visualized with fluorescence microscopy. Images were captured using a high-resolution camera and analyzed using MetaMorph software (Molecular Probes, Carlsbad, CA) for 90 minutes at 37°C in DMEM containing 2% FBS. Before analysis, cells were washed and resuspended in 2 μg/mL propidium iodide (Sigma, St. Louis, MO). Cells were analyzed on a LSR flow cytometer equipped with 424/44-nm band pass and 670-nm long pass optical filters (Omega Optical, Brattleboro, VT). To ensure proper identification of side population cells, cells were incubated as above with the addition of 30 μM/L verapamil (Sigma).

Real-time reverse transcription-PCR. Quantitative reverse transcription-PCR (RT-PCR) for Notch1, Notch2, and Hes1 was done as previously described (31), with all reactions normalized to actin (Applied Biosystems, Foster City, CA). Commercially available Assay on Demand TaqMan primers and probes were used to measure mRNA for Tuj1 and the α subunit of the γ-aminobutyric acid type A receptor (GABRA6). Each quantitative RT-PCR reaction was done in triplicate and error bars represent SE.

Protein analysis. Western blots contained 20 μg of protein per lane on a 10% Tris-glycine SDS-PAGE gel (Invitrogen) and electrophoresed for several hours in 1× TG-TDS buffer (Amresco, Solon, OH). Proteins were transferred to 0.45-μm Optitran nitrocellulose (Schleicher & Schuell, Keene, NH) in 1× Tris-glycine buffer (Amresco). Blots were blocked in PBS containing 5% nonfat dry milk powder and incubated overnight at 4°C with antibodies directed against Hes1 (kind gift of Dr. Tetsuo Sudo, Toray Industries, Teibiro, Kamakura, Japan; 1:2,000) or glyceraldehyde-3-phosphate dehydrogenase (Research Diagnostics, Flanders, NJ; 1:20,000). Blots were then washed several times with PBS containing 0.1% Tween 20 and incubated in peroxidase-conjugated IgG diluted 1:2,000 in blocking solution. After washing several times in PBS with 0.1% Tween 20, blots were developed with enhanced chemiluminescence reagent (Pierce, Rockford, IL) and exposed to film.

Statistical analysis. Statistical analyses were done using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Data graphed with error bars represent mean and SE from experiments done in triplicate unless otherwise noted. A two-sided Student’s t test was used to determine the significance of any differences.

Results

Growth of medulloblastoma cultures is slowed but not arrested by Notch blockade. Experiments were done using GSI-18, a potent γ-secretase inhibitor with a sulfonamide core (39). We first sought to determine what concentration of GSI-18 effectively inhibited Notch activity in tumor cells by measuring expression of the pathway target Hes1. GSI-18 at 2 μM/L reduced both mRNA and protein levels of Hes1 in DAOY cells by >70% (Fig. 1A and B), suggesting that this concentration should be sufficient to cause antitumor effects mediated by Notch pathway inhibition. GSI-18 levels <0.4 μM/L did not affect Hes1 mRNA expression (data not shown).

Notch pathway inhibition using GSI-18 slowed the growth of DAOY medulloblastoma cultures. The increase in viable cell mass over 2 days was reduced in a dose-dependent fashion by GSI-18 (Fig. 1C) but many tumor cells survived Notch pathway inhibition and continued to proliferate over this period. JC2, a γ-secretase inhibitor with a benzodiazepine core, also slowed but did not arrest growth in medulloblastoma cell mass when used at concentrations that effectively inhibited the Notch pathway, indicating the findings were not specific to one structural class of compound (data not shown). DAOY cells stably transfected with a NICD2 were used to further control for the specificity of the pharmacologic effects. This truncated Notch receptor does not require ligand binding or γ-secretase activity for nuclear translocation and signaling, and cells expressing it should be insensitive to effects of GSI-18 mediated by Notch2 inhibition. NICD2 expression in a stable subclone we have previously described (8) rescued the negative effects on DAOY cell mass (Fig. 1D). A second stable subclone, as
well as a pool of cells into which NICD2 was introduced by transfection, was also resistant to the growth-inhibiting effects of GSI-18 (data not shown), suggesting that this compound acts through Notch and not other pathways regulated by γ-secretase.

It has previously been reported that in pre-T cells, downregulation of Notch activity reduces cellular metabolism and cell size (41). We also observed decreased cell size in medulloblastoma cultures following GSI-18 exposure (Fig. 1E). To rule out the possibility that our measurements of viable cell mass bioreductive capacity (CellTITER assay) were being altered by metabolic affects of Notch, we quantitated cell number using flow cytometry. The number of live cells in DAOY, D283Med, D425Med, and PFSK cultures exposed to 2 μmol/L GSI-18 for 48 hours was reduced as compared with vehicle-treated controls, but the reduction was less than that of cell mass (Fig. 1F). Thus, measures of both cell mass and cell number show a slowing, but not an arrest, of tumor growth under conditions of Notch blockade.

**Decreased proliferation and increased neuronal differentiation following Notch inhibition.** Flow cytometric analysis showed that 2 μmol/L GSI-18 increased the G1-G0 cell fraction and decreased the S-phase and G2-M fractions of DAOY and PFSK cell lines (Fig. 1G), suggesting that cell cycle exit likely plays a role

![Figure 1](image)
in its antigrowth effects. Notch pathway down-regulation has also been linked to cellular differentiation in both normal development and in neoplasms (42, 43) and we therefore examined whether Notch inhibition caused cellular differentiation. γ-Secretase inhibition in DAOY cells increased RNA levels of two markers of cerebellar neuronal differentiation, TuJ1 and GABRA6, in a dose-dependent fashion (Fig. 1H). One of these neuronal markers, GABRA6, is specifically found in cerebellar granule cells in the brain, where its expression is induced as they mature (44). Its induction after Notch pathway inhibition suggests that the differentiation pathway being actuated resembles that in normal cerebellar granule neuron precursors, and highlights the similarities between the DAOY line and developing cerebellum.

**Notch blockade by γ-secretase inhibitors suppresses tumor formation.** We next examined the effects of Notch pathway blockade on tumor formation in vitro and in vivo. We first used a clonogenic assay to determine whether cells capable of forming anchorage-independent colonies were depleted by GSI-18. Vehicle-treated DAOY cultures seeded in soft agar formed 50 colonies per field on average (Fig. 2). This number dropped to 4 when an equal number of viable cells was counted and seeded after 48 hours of treatment with GSI-18 and increased to 119 in the presence of constitutive Notch2 activation. Thus, whereas many tumor cells continued to grow in 2 μmol/L GSI-18, their clonogenicity in soft agar was suppressed by >90%.

To determine the effects of Notch blockade on the formation of tumor xenografts, DAOY and DAOY:NICD2 cultures were treated with either 2 μmol/L GSI-18 in DMSO or DMSO alone, and the viable cells remaining after 48 hours were counted using trypan blue. Five hundred thousand viable cells from each group were then mixed with Matrigel and injected s.c. in athymic (nude) mice. Large xenografts formed at the sites of all 12 control injections, including vehicle-treated DAOY cells (n = 4; Fig. 3A), vehicle-treated DAOY:NICD2 cells (n = 4), and GSI-18-treated DAOY:NICD2 cells refractory to Notch inhibition (n = 4). In contrast, only one very small lesion formed among the four sites injected with 500,000 DAOY cells that had been pretreated with 2 μmol/L GSI-18, suggesting that while viable, the cancer cells were no longer...
Gain or loss of Notch pathway activity modulated the size of the CD133-expressing stem-like subpopulation (Fig. 4B). This fraction was elevated from 9.9% to >17% in a pool of DAOY cells transfected with a plasmid encoding the constitutively active NICD2 and in two stable subclones derived from such a pool. In contrast, Notch pathway inhibition using GSI-18 reduced the CD133-positive stem-like fraction 3-fold to 3.3% (P < 0.01). As expected, NICD2 expression rescued the effects of GSI-18 on the CD133-positive cell fraction. A 4.8-fold reduction in the percentage of CD133-positive cells seen in PFSK cultures was also statistically significant (P < 0.01). Decreases in the stem-like fraction were also observed in the D283Med and D425Med lines but were less pronounced.

Side population was analyzed to obtain a second measure of the effects of Notch on stem-like tumor cells. DAOY cultures contained a Hoechst 33342-excreting side population of 1.9% (Fig. 5A). The multiple drug resistance pumps mediating dye efflux are verapamil sensitive (28), and adding verapamil to the medulloblastoma culture abolished Hoechst excretion, verifying that this was a bona fide side population. Consistent with our hypothesis that Notch activity regulates stem-like cancer cells, constitutive activation of Notch2 increased the side population almost 4-fold to 7.3%, whereas Notch inhibition essentially ablated it, with only 0.01% of remaining cells negative for dye (Fig. 5A and B). NICD2 expression protected the side population from GSI-18 exposure, but not from verapamil, indicating that the effect of GSI-18 was specifically mediated through its effects on Notch signaling. Small side populations were also present in the PFSK, D283Med, and D425Med cell lines and were ablated by both verapamil (data not shown) and 2 μmol/L GSI-18 (Fig. 5B). Thus, Notch pathway blockade depletes the stem-like subpopulation defined either by CD133 or by side population.

**Figure 4.** The CD133-positive population in medulloblastoma is regulated by Notch. A, flow cytometric analysis was used to define the population of cells with CD133 expression elevated above the highest level of background fluorescence (dashed line). Cultures were evaluated following 48-hour exposure to either 2 μmol/L GSI-18 or vehicle. B, the CD133-positive subpopulation in all four medulloblastoma/PNET lines examined was reduced by 2 μmol/L GSI-18 (*, P < 0.01). Constitutive activation of Notch2 in a pool of DAOY cells transfected with NICD2 (P) as well as in two stable subclones (C1 and C2) rendered the CD133-expressing subpopulation insensitive to γ-secretase inhibitor. C, Hes1 mRNA levels were elevated in CD133-enriched preparations as compared with CD133-depleted ones, suggesting Notch activity is higher in stem-like cells.
Notch blockade induces apoptosis in nestin-positive medulloblastoma cells. We next examined if stem or progenitor-like cells expressing nestin might be especially prone to apoptosis following Notch pathway blockade. Expression of the intermediate filament nestin was used to directly visualize poorly differentiated cells, as CD133 immunofluorescence was too dim to reliably score by eye. Nestin mRNA levels were not significantly elevated in CD133-enriched DAOY populations, suggesting that the two markers are not equivalent (data not shown). This is not unexpected, as Lee et al. (45) also found that only 20% of CD133-positive cells in the postnatal cerebellum also expressed nestin. Nevertheless, nestin was one of the first markers of neural stem cells to be identified (46) and its expression has been shown in a subset of cells within neurospheres derived from primary human medulloblastoma, suggesting it marks stem-like cells in these tumors (32, 33). We detected nestin protein in both established (DAOY) and low-passage (MB2) medulloblastoma cultures. Nestin staining was present in the cytoplasm and was variable in intensity, with only 10% of DAOY cells expressing high levels whereas almost half of MB2 cells were strongly nestin positive (Fig. 6A and B). We reasoned that medulloblastoma cells expressing high levels of nestin might represent a stem-like subpopulation and we therefore correlated their percentage with varying levels of Notch activity. In DAOY cells, constitutive Notch2 activation up-regulated the strongly nestin-positive fraction of cells almost 5-fold to 47.7% (P < 0.001) whereas Notch blockade using the γ-secretase inhibitor GSI-18 reduced this population 4-fold to 2.4% (P < 0.01; Fig. 6C). A significant reduction was also observed in MB2 cells following Notch blockade.

We used double immunofluorescence to study cell death in medulloblastoma cells positive or negative for nestin. The basal apoptotic rate was measured using antibodies specific for cleaved caspase-3. In DAOY cultures to which vehicle was added for 48 hours, the apoptotic rate was low in both nestin-negative (26 of 1,841; 1.4%) and nestin-positive (4 of 160; 2.5%) cells (Fig. 6D-F). However, after 48 hours of treatment with 2 μmol/L GSI-18, the apoptotic rate increased to 37% in the nestin-positive population (23 of 62 cells) whereas apoptosis in the better-differentiated cells lacking nestin only increased to 3.9% (40 of 1,017 cells). Interestingly, nestin was less evenly distributed in the apoptotic cells and was sometimes observed surrounding the degenerating nuclei. Such cagelike perinuclear nestin staining has been previously described in mitotic cells (47). Our immunofluorescent assessment of cell death using cleaved caspase-3 corresponds relatively well to apoptotic induction measured by flow cytometric analysis of Annexin staining, suggesting that our manual counting procedure is accurate (Fig. 6G). Taken together, these data indicate that the survival of stem-like tumor cells is more sensitive to Notch pathway inhibition than the survival of better-differentiated cells.

Discussion

In this study, we examined the effects of Notch pathway inhibition on the growth and xenograft formation of medulloblastoma. We found that Notch blockade using the γ-secretase inhibitor GSI-18 slowed the growth of tumor cells in vitro but had a much more dramatic effect on the formation of colonies in soft agar and tumor xenografts in nude mice. Indeed, our most striking finding was that large numbers of viable, rapidly proliferating cells were not able to generate bulky xenografts if they had previously been treated with GSI-18, whereas equal numbers of vehicle-treated cells always formed large tumors. Systemic (i.p.) treatment of animals with GSI-18 also blocked xenograft formation with no apparent side effects, indicating this agent may be effective therapeutically in preventing tumor metastasis or regrowth following debulking. We believe that these dramatic effects on tumor propagation are mediated by depletion of cancer stem cells, as subpopulations expressing the stem cell marker CD133, as well as the stem-like side population, were profoundly reduced by Notch blockade. These results must be extended to primary tumors or additional low-passage lines and might provide a first indication that stem-like cells can be successfully ablated from brain tumors.

The targeted depletion of stem-like cells we observe following Notch pathway blockade contrasts sharply with prior reports of cancer stem cell survival following standard chemotherapies. For example, Wulf et al. (48) showed higher efflux of chemotherapeutic agents and better survival in leukemic side population cells as compared with non-side population cells. Based on this and on similar studies, it has been suggested that the efflux pumps which define side population also function to remove toxic chemotherapy drugs from cancer stem cells (reviewed in refs. 49, 50). In support of this concept, treatment of neuroblastoma cell lines with mitoxantrone actually increases the side population, suggesting that stem-like cancer cells are relatively resistant to this chemotherapeutic agent and accumulate as more differentiated cells are killed (51). Thus, conventional chemotherapies effectively remove the better-differentiated cells while leaving most stem-like cells alive. In contrast, Notch blockade depletes stem-like cells but leaves many better-differentiated cells capable of limited growth intact.
We examined only one type of malignant brain tumor in this study, but our findings may be applicable to other neoplasms as well. The Notch pathway is known to regulate stem cells in a wide variety of tissues and Notch blockade seems to affect survival and proliferation of multiple types of cancer (52, 53). For example, Notch is activated by translocation or mutation in >50% of T-cell acute lymphoblastic leukemia, and anti-Notch therapies have been shown to slow acute lymphoblastic leukemia growth \textit{in vitro} (38). Aberrantly activated Notch signaling has also been documented in lung, breast, salivary gland, and pancreatic carcinoma (54–57). γ-Secretase inhibitors may therefore be useful in targeting stem-like cancer cells in a wide range of neoplasms.

In the future, multiagent chemotherapeutic regimens may target both stem-like and better-differentiated cells. Drugs blocking Notch signaling, or other pathways required in stem cells such as Wnt and Hedgehog, will be used to deplete the cancer stem cell population, and traditional chemotherapeutic agents can be used at the same time to debulk the larger mass of tumor cells. This will effect a rapid removal of both subpopulations and might circumvent the possibility that some differentiated tumor cells can dedifferentiate and repopulate the stem cell fraction (58). Another potential complication of cancer stem cell–directed therapies is that nonneoplastic stem cells may also be depleted by such strategies. This is of particular concern in children who will presumably require stem cells for the maintenance and repair of a host of tissues over the course of their lives. Of note, we found that i.p. injections of GSI-18 for 5 days blocked tumor formation with no obvious ill effects on the animal’s health over the subsequent months. Indeed, it has recently been shown that inhibition of γ-secretase activity using small-molecule drugs can actually enhance long-term memory in rodents (59).

In summary, we show that in the malignant brain tumor medulloblastoma, Notch pathway blockade depletes a population of cells required for \textit{in vivo} tumor formation by suppressing

![Figure 6](image)

**Figure 6.** Nestin-positive cells are especially sensitive to Notch pathway blockade. A, the fraction of DAOY cells expressing high levels of nestin is lower after GSI-18 exposure and higher in a subclone expressing activated Notch2 (NICD2). B and C, similar effects are seen following Notch blockade in the low-passage medulloblastoma cell line MB2. D to F, double immunolabeling was used assess both differentiation status (nestin) and apoptosis (cleaved caspase-3) in DAOY cultures. Significant induction of cleaved caspase-3–positive apoptotic cells following Notch blockade was limited to the nestin-positive subpopulation. G, overall apoptotic induction in DAOY cultures was similar when measured by counts of cleaved caspase-3 immunofluorescent (IF) cells or flow cytometric analysis of Annexin.*, \( P < 0.01; \) **, \( P < 0.001 \) (t tests); ns, not significant.
proliferation and inducing apoptosis or differentiation in stem-like cells. Our data suggest that Notch pathway blockers may be the first of a new class of chemotherapeutic agents—those targeting cancer stem cells.

Acknowledgments

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References


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