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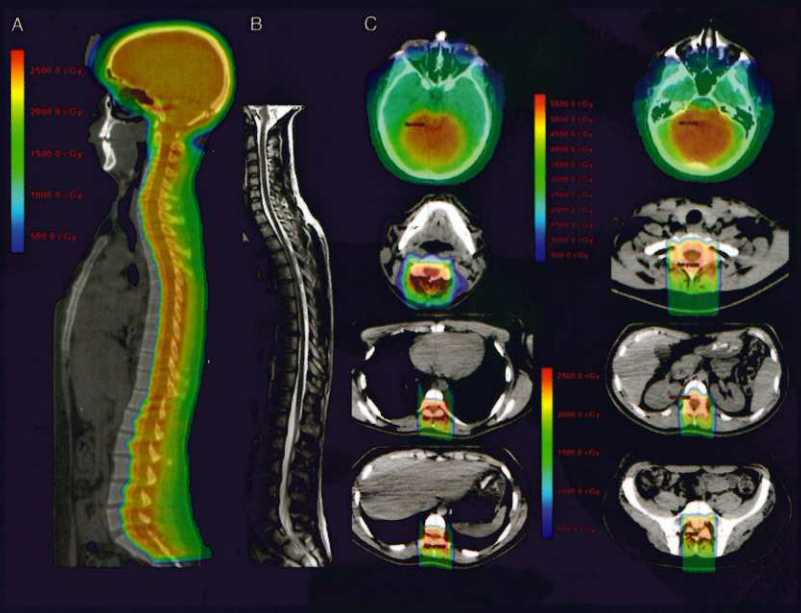

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Differential patterns of NOTCH1–4 receptor expression are markers of glioma cell differentiation

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Background. Notch signaling is deregulated in human gliomas and may play a role in their malignancy. However, the role of each Notch receptor in glioma cell differentiation and progression is not clear. We examined the expression pattern of Notch receptors and compared it with differentiation markers in glioma cell lines, primary human cultures, and biopsies of different grades. Furthermore, the effects of a γ-secretase inhibitor (GSI) on cell survival were assessed.

Methods. Notch receptors and markers of cellular differentiation were analyzed by reverse transcriptase PCR, Western blotting, immunohistochemistry, and immunocytochemistry. GSI sensitivity was assessed in both cell lines and primary cultures grown as monolayers or tumorspheres, by MTT assay.

Results. In cell lines, Notch1 and Notch2/4 levels paralleled those of glial fibrillary acidic protein (GFAP) and vimentin, respectively. In human gliomas and primary cultures, Notch1 was moderate/strong in low-grade tumors but weak in glioblastoma multiforme (GBM). Conversely, Notch4 increased from astrocytoma grade II to GBM. Primary GBM cultures grown in serum (monolayer) showed moderate/high levels of CD133, nestin, vimentin, and Notch4 and very low levels of GFAP and Notch1, which were reduced in tumorspheres. This effect was drastic for Notch4. GSI reduced cell survival with stronger effect in serum, whilst human primary cultures showed different sensitivity.

Conclusion. Data from cell lines and human gliomas suggest a correlation between expression of Notch receptors and cell differentiation. Namely, Notch1 and Notch4 are markers of differentiated and less differentiated glioma cells, respectively. We propose Notch receptors as markers of glioma grading and possible prognostic factors.

Keywords: cell differentiation, gene expression, gliomas, glioma cell cultures, intermediate filaments, Notch receptors.

Gliomas represent the most frequent primary tumors of the CNS. The invasive nature of these malignant neoplasms most often determines the inability of surgery to cure patients. Gliomas are currently classified according to their hypothesized line of differentiation (eg, astrocytes, oligodendrocytes, ependymal cells) and are usually grouped into 4 World Health Organization (WHO) clinical grades according to their degree of malignancy. Grade I tumors are biologically "benign" and can be surgically cured, if resectable, at the time of diagnosis; grade II tumors are low-grade malignancies with long clinical courses, which are not curable by surgery. Grades III and IV are malignant gliomas, the first of which leads to death within a few years, while the highly malignant grade IV, not curable with chemotherapy, is lethal within 9–12 months.[1] Treatment of CNS tumors will be fueled by discovering connections with genes that control cell growth, proliferation, differentiation, and death during normal development. In addition, it will be of great importance to evaluate proteins that could be considered markers possibly involved in tumorigenic key events.[2]

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Among these, mammalian Notch genes are interesting candidates because they are implicated in cell fate decision, proliferation, and differentiation of many cell types during embryonic development and are also involved in tumorigenesis.

The Notch family of transmembrane receptors comprises Notch1, -2, -3, and -4.[3] Mature Notch receptors are heterodimers derived from the cleavage of Notch pre-proteins into an extracellular subunit containing multiple Epidermal Growth Factor (EGF)–like repeats and a transmembrane subunit including the intracellular region.[4] In humans, Notch receptors recognize 5 ligands: Delta-like 1, Delta-like 3, Delta-like 4, Jagged-1, and Jagged-2.[5] The interaction between one of these ligands and a Notch receptor triggers 2 successive cleavages, the first mediated by the Tumor Necrosis Factor–α converting enzyme (TACE) and the second by γ-secretase, generating an intracytoplasmic fragment of Notch (Notch intracellular domain [NICD]). NICD translocates into the nucleus, where it binds to the transcription factor CBF1/Su(H)/LAG1 (CSL). This interaction results in the displacement of a co-repressor and recruitment of a co-activator leading to transcriptional activation of target genes. In fact, Notch signaling activates a diverse repertoire of genes, the products of which can activate or inhibit many different cellular functions. Notch signaling has been referred to as a "gatekeeper against differentiation."[6] It can alternatively force the cell to remain in an undifferentiated state, drive cells toward a primary differentiation fate blocking it, or direct the cell to a second alternative differentiation program. In the nervous system, Notch promotes the differentiation of various glial cell types, including astrocytes,[7] Schwann cells,[8] Müller glial cells,[9] and radial glial cells.[10] Notch receptor expression patterns have been studied in mammalian brain during development and in postnatal life. Notch1, -2, and -3 mRNAs have been observed in late embryonic and postnatal rat brain by in situ hybridization. Notch1 mRNA is present in regions where neurogenesis continues in postnatal brain,[11] whereas Notch2 and Notch3 mRNAs are highly expressed in the ventricular zones.[12] After the embryonic period, Notch1 and Notch2 are still expressed in specific regions of postnatal brain, while Notch2 is expressed in cerebellar Bergmann glia and in hippocampal radial glia.[13] Notch2 is predominantly expressed in proliferating progenitors[14] and Notch1 in postmitotic differentiating cells. Moreover, while Notch1 and Notch2 are broadly expressed in most tissues such as brain, liver, heart, lung, and other organs and sometimes have overlapping expression, Notch3 and Notch4 have a more restricted expression. In fact, Notch3 is expressed in the CNS,[15] in vascular smooth muscles,[16] and in certain hematopoietic cells, while Notch4 is expressed in vascular endothelial cells preferentially.[17]

Depending on the cell type, Notch can work as an oncogene or as a tumor suppressor.[18,19] Furthermore, Notch signaling deregulation could be involved in the genesis of different cancer types. Recent data suggest that Notch signaling is deregulated in several brain tumors and potentially involved in glioma malignancy (for a review, see Stockhausen et al.[20]). Notch1 and its ligands are expressed in human gliomas, and Notch1 downregulation results in inhibition of cell survival and proliferation.[21] Furthermore, Notch2 may play a role in gliomagenesis[22] and has been indicated as a negative prognostic marker in human glial brain tumors.[23] Interestingly, Notch3 activation promotes invasive glioma formation in the optic nerve but not in the brain, suggesting that each Notch paralog plays a tumorigenic role in a context-dependent manner.[24]

In the present study, we examined the expression of Notch1–4 receptor paralogs in glioma cell lines (U87 and U373 from human and C6 and 9L from rat) compared with primary astroglial cultures derived from newborn rat cerebral hemispheres, in primary human glioma cell cultures (low- and high-grade glioma cultures), and in human glioma biopsies of different WHO grades. Our aim was to find a correlation between Notch receptor expression and glioma grading and more importantly to individuate possible markers of glioma cell differentiation. The expression of Notch receptors in cell lines and primary cell cultures grown in serum or as neurospheres was examined, and the effect of γ-secretase inhibitor (GSI) on glioma cell survival in these culture conditions was also evaluated.

Materials and Methods

Cell Cultures

Primary astroglial cell cultures were prepared from cerebral hemispheres of newborn rats as previously described.[25] In brief, the meninges were removed and cerebral hemispheres passed through a sterile nylon sieve (pore size 82 μm) into nutrient medium. The basal nutrient medium consisted of Dulbecco modified Eagle's medium (DMEM; Gibco/Invitrogen) containing 10% heat-inactivated fetal calf serum (FCS; Gibco/Invitrogen), 2 mM glutamine, and penicillin (50 U/mL) and streptomycin (0.05 mg/mL). Cells were seeded into plastic Falcon petri dishes at a plating density of 0.5×10^5 cells/cm^2. Cultures were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere, and the medium was changed after 6 days and then twice a week. Cultures were used at 8 and 13–15 days in vitro (DIV).

Rat glioma cell lines C6 and 9L and human U87 and U373 were grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Gibco/Invitrogen) containing 10% FCS, 4 mM glutamine, and penicillin (50 U/mL) and streptomycin (0.05 mg/mL). Cultures were used at 8 and 13–15 DIV. Glioma cell lines were a kind gift of Dr G. Finocchiaro, Neurological National Institute Besta in Milan.

Primary human glioma cell cultures

Following informed consent, tumor samples classified as grades II, III, and IV or glioblastoma multiforme (GBM) based on WHO criteria[1] were obtained from patients who underwent surgical resection at the Neurosurgery division of the Department of Neurosciences, University of Catania. Biopsies were processed within 1 to 2 h after surgical removal. Tumors were washed with Hank's Balanced Salt Solution and enzymatically dissociated into single cells. Red blood cells were removed by differential centrifugation. Tumor cells were cultured in either DMEM-F12 (1:1) media (Invitrogen) plus N2 and B27 supplements (0.5× each; Invitrogen), human recombinant basic Fibroblast Growth Factor (bFGF) and EGF (20 ng/mL each; Peprotech), which allowed neurosphere formation (NSF), or serum media consisting of DMEM-F12 (1:1) with 10% heat-inactivated FCS. In addition, primary cultures were seeded in the presence of 10% FCS and then either grown in the presence of FCS or shifted to NSF. The histopathological features of fresh resected glial tumors used for primary cultures are reported in Table 1.

Reverse Transcriptase PCR

Total RNA was extracted from cell cultures of rat and human glioma cell lines (C6, 9L, U87, and U373) and from rat primary astrocyte cell culture by using Total RNA Isolation Reagent (TRIzol, Gibco), as indicated by the manufacturer. RNA obtained was dissolved in RNAse-free water. Total RNA (1 μg) was added to 50 ng/μL oligodeoxythymidine, deoxyribonucleotide triphosphate mix (1 mM of each), dissolved in diethyl pyrocarbonate–

Table 1. Histopathological features of human glial tumors

Specific Diagnosis	Number	Location
Gliomas used for primary cultures		
Astrocytoma II	3	Frontal: 1; temporal: 1; Hippocampal: 1;
Anaplastic astrocytoma	2	Frontal: 1; parietal: 1;
GBM	6	Frontal: 2; temporal: 3; Hippocampal: 1
Gliomas used for immunohistochemistry		
Astrocytoma II	6	Frontal: 3; parietal: 2; temporal: 1
Anaplastic astrocytoma	6	Frontal: 4; parietal: 2
GBM	6	Frontal: 3; parietal: 2; temporal: 1

Table 2. PCR primers specifically targeting Notch1–4 receptors

Gene Name	Primers		
	Sense	Antisense	bp
NOTCH-1	GCTACAACTGCGTGTGTGTC	GTTGGTGTCGCAGTTGGAGC	218
NOTCH-2	CACCTTGAAGCTGCAGACAT	GGTAGACCAAGTCTGTGATG	217
NOTCH-3	ACTGGACCTCGCTGTGAGAC	GCAGCTGAAGCCATTGACTC	210
NOTCH-4	CAGCTGCCTTGATCTTCC	CTTGAGTGACAAGCTGTT	402
NOTCH-4 (H)	GCTATGTGTCTCAGTGGTCA	AAGCTTGGCCTGGCATCTCT	310
GFAP	GATGATGGAGCTCAATGACC	GATCTCCTCCTCCAGCGACT	376
GAPDH	TCACTGGCATGGCCTTCCGT	CTTACTCCTTGGAGGCCAT	327

treated water. Two microliters of reaction buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), $MgCl_2$ (5 mM), and dithiothreitol (10 mM) were added. The reverse transcription of total RNA was performed at 42°C for 50 min using 1 μL of Superscript II RT (50 U/μL; Invitrogen) followed by 15 min at 70°C. Samples were further incubated at 37°C for 20 min with 1 μL of RNAse H (2 U/μL). Complementary DNAs obtained were stored at −20°C. As a control for genomic DNA contamination, identical reactions were performed without reverse transcriptase (RT) enzyme. The cDNA obtained was used to perform PCR reactions. The primers reported in Table 2 were purchased from MWG Biotech and used for PCR. Amplified DNA fragments were then electrophoresed through agarose gels and visualized by ethidium bromide.

Western Blot Analysis

Cells were lysed using a lysis buffer containing Tris-HCl (1 M, pH 6.8), Leupeptin (10 μg/mL), Aprotinin (5 μg/mL), phenylmethylsulfonyl fluoride (50 μM), EDTA (0.5 M), EGTA (0.5 M), phosphatase inhibitor cocktail II (5 mg/mL, Sigma-Aldrich), and sodium dodecyl sulfate (SDS) 10%. Protein concentration was determinated by the bicinchoninic acid method (BCA Protein Assay Kit, Pierce). For western blot analysis, 100, 50, or 25 μg of total proteins were separated by 8% SDS-polyacrylamide gel electrophoresis and then transferred onto 0.45 μm supported nitrocellulose membrane (Bio-Rad) at 350 mA for 90 min using a Mini Trans-Blot Cell apparatus (Bio-Rad). Filters were blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline (TBS) and Tween 20 (19.9 mM Tris base, 136 mM NaCl, 0.1% Tween 20, pH 7.6) and incubated overnight at 4°C with the following primary antibodies: anti-Notch1 rabbit polyclonal (Upstate Biotech), anti-Notch2 goat polyclonal (Santa Cruz Biotechnology), anti-Notch3 goat polyclonal (Upstate Biotech), and anti-Notch4 rabbit polyclonal (Santa Cruz Biotechnology) diluted at 1:2000, 1:400, 1:200, and 1:200, respectively. The antibodies anti-actin goat polyclonal (Santa Cruz Biotechnology), anti–glial fibrillary acidic protein (GFAP) monoclonal (Immunological Sciences), anti-vimentin monoclonal (Dako), anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal (Chemicon), anti-Hes1 polyclonal (Chemicon), anti-nestin polyclonal (Abcam), and anti-CD133 polyclonal (Abcam) were used at dilutions of 1:1000, 1:500, 1:25, 1:1000, 1:200, 1:500, and 1 μg/mL, respectively. Anti-rabbit (Upstate Biotech), -goat (Santa Cruz Biotechnology), and -mouse (Amersham) secondary antibodies linked to horseradish peroxidase and alkaline phosphatase were used. Immunoreactivity was detected using enhanced chemiluminescence (ECL Plus, Amersham) and the WesternBreeze Chemiluminescent Western Blot Immunodetection Kit (Invitrogen). Autoradiographic signals were evaluated by densitometric analysis.

Cellular Viability of Glioma Cultures After GSI Treatment

Cells were seeded in plastic 96-multiwell plates (Nunc) at a cellular density of 2000/3000 cells/well and cultured for different times. Primary cultures were used at passages 3–5.

The colorimetric assay MTT[26] based on the use of the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was used to evaluate both cell proliferation at 1, 7, 14, 21, 28, 35, and 42 DIV and cellular viability after 24 h treatment with 1, 5, 10, 25, and 50 μM GSI (Calbiochem/Inalco) dissolved in dimethylsulfoxide. Controls in dimethylsulfoxide were included in experimental conditions. Apoptosis was evaluated by a caspase-3 activation assay. Cells were treated with GSI (10 μM) or with GSI (10 μM) plus Z-VAD-FMK (50 μM), a pan-caspase colorimetric inhibitor (CaspACE Assay System, Promega) at the same time as GSI. The activity of caspase-3 was revealed after 24 h by a multiscan reader at 405 nm.

Subjects

The cases included in the immunohistochemistry analysis in this study were obtained from the files of the Department of Neuropathology of the Academic Medical Center at the University of Amsterdam. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tumor samples from 18 patients with primary glial tumors who underwent surgical resection were included in the study for immunohistochemical analysis of Notch1–4. These were 6 grade II astrocytomas, 6 anaplastic astrocytomas, and 6 GBM (see Table 1). All cases were reviewed independently by 2 neuropathologists and the diagnosis confirmed according to the revised WHO classification of tumors of the CNS.[1] Control brain tissue (including normal cortex and white matter from the temporal, frontal, and parietal regions) was obtained from 6 age-matched patients who died from a nonneurological disease.

Tissue Preparation

All specimens used in the study for immunohistochemistry were fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6 μm on a sliding microtome and mounted on slides coated with organosilane (3-aminopropylethoxysilane; Sigma). Representative sections of all specimens were processed for hematoxylin and eosin staining, as well as for immunohistochemical reactions.

Immunohistochemistry

To detect Notch proteins, we used polyclonal rabbit anti-Notch1 (1:70; Upstate Biotech) and polyclonal goat anti-Notch2, anti-Notch3, and anti-Notch4 (1:20; Santa Cruz Biotechnology). Immunohistochemistry in sections of routinely processed formalin-fixed, paraffin-embedded tissues

Table 3. Synopsis of Notch1, Notch4, and Hes1 expression levels and percentage of Ki-67 in human normal brain and in glioma biopsies

Tissue	Grade	Subject Reference	Notch1	Notch4	Hes1	% Ki-67
Normal	N	*WM 160*	+/−	−	−	ND
		WM 162	+/−	−	−	ND
		WM 066	+/−	−	−	ND
		WM 158	+/−	−	−	ND
Low-grade gliomas	II	*CM50**	+	−	+/−	4
		RC39	+	ND	ND	4
		10353	+	++	+/−	<1
		07611	+	+	+/−	2
		14144	+	++	+	3
		15578	++	++	+/−	2
High-grade gliomas	IV	*CAM50*	+/−	++	ND	40
		*BM38**	+/−	+	++	40
		*GS51**	+/−	+	+	−
		*MS81**	+/−	++	+/−	30
		LE38	+/−	+	ND	30
		AGP48	+/−	++	ND	>60
		*FL75***	+/−	+	+	50
		01616	+/−	++	+/−	20
		09851	++	++	+/−	20
		10034	+/−	++	−	15
		06918	++	++	+/−	10

Abbreviations: ND, not determined; − = absent, +/− = low, + = moderate, ++ = strong.
*Biopsy where primary cultures were obtained and analyzed through western blot analysis and MTT assay.
**Biopsy where primary cultures were obtained and analyzed through western blot analysis, MTT assay, and immunocytochemistry.

was carried out as previously described.[27,28] Single-label immunohistochemistry was performed using the avidin-biotin peroxidase method (Vector Elite) and 3,3′-diaminobenzidine as chromogen. Sections were counterstained with hematoxylin. Sections incubated without the primary antibody or with preimmune sera were essentially blank.

Evaluation of Immunostaining

All labeled tissue sections were evaluated by 2 independent observers, with respect to the presence or absence of various histopathological parameters and specific immunoreactivity for the different markers. The intensity of Notch1–4 staining was evaluated on a scale of 0–3 (0 = no, 1 = weak, 2 = moderate, 3 = strong). All areas of the specimen were examined, and the score represented the predominant cell staining intensity found in each case. The frequency of positive cells (1< 1%; 2, 1-10 %, 3, 11–50 %; 4, > 50%) was also evaluated to give information about the relative number of positive cells within the specimen. As proposed before,[28,29] the product of these 2 values (intensity and frequency scores) was taken to give the overall score (total score) shown in Fig. 3.

Immunocytochemistry

Primary human glioma cell cultures were used for immunocytochemical procedures. Cells were fixed by exposure to 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 10 min. After washing in TBS, the cell membranes were permeabilized with TBS containing 0.2% Triton X-100 (TBS-T) at room temperature for 15 min. Following blocking with 5% normal goat serum (NGS) in 0.2% TBS-T at room temperature for 45 min, cultures were incubated overnight at 4°C in the following primary antibodies: anti-Notch1 (1:100 anti-rabbit; Upstate), anti-Notch4 (1:100 anti-rabbit; Santa Cruz), anti-CD133 (1:200 anti-rabbit; Abcam), anti-nestin (1:200 anti-rabbit; Abcam), anti-GFAP (1:1000 anti-rabbit; Dako), and anti-vimentin (1:100 anti-mouse, Dako). All primary antibodies were diluted in TBS-T containing 1% NGS. After washing in TBS, cultures were incubated for 2 h at room temperature with correspondent anti-rabbit or anti-mouse Cy3 (1:200; Immunological Science) diluted in TBS-T containing 1% NGS. Sytox dye (1:10 000; Molecular Probes) was used for nuclear counterstaining, and finally coverslips were mounted in FluorPreserve Reagent (Calbiochem). Primary antibody controls were performed by eliminating incubation for each primary antibody. Images were collected on a confocal laser scanning microscope (Zeiss LSM 510 Meta).

Ki-67 Immunohistochemical Analysis

The evaluation of the proliferative index Ki-67 was performed in a set of glioma biopsies (Table 3) by immunohistochemical analysis on 5-μm-thick formalin-fixed, paraffin-embedded tissue sections using MIB-1, a monoclonal antibody directed against the Ki-67 antigen (1:75, Dako). Immunohistochemical studies were performed with the labeled streptavidin-biotin peroxidase detection system using the Ventana automated immunostainer. Briefly, the sections were deparaffinized and dehydrated in a series of "dewax" solutions and alcohol. Heat-induced antigen retrieval was performed with a high pH antigen retrieval buffer (ER2). After washing with PBS 3 times for 5 min each, the slides were incubated with 0.5% H_2O_2 for 20 min to quench endogenous peroxidase. Normal horse serum at a dilution of 1:50 was added to each slide to block nonspecific reactions and slides were incubated for 20 min. Incubation with primary antibody was followed by incubation with a biotinylated rabbit secondary antibody at a dilution of 1:200 for 45 min and 3 washes with PBS and substrate. The positive results were visualized with 3,3′-diaminobenzidine. Finally, the sections were counterstained and coverslipped. Any nuclear

staining was considered indicative of Ki-67 expression. The control slide (without the primary antibody) was used to ensure that protocols were followed correctly. MIB-1 labeling index was evaluated in the highest immunoreactivity fields, expressed as percentage of positive staining nuclei, and determined after counting at least 1000 tumor cells.

Results

Glioma Cell Lines Express Different Levels of Vimentin and GFAP

The first analysis was designed to examine the cellular differentiation of the 4 glioma cell lines through the expression of proteins, such as vimentin and GFAP, which are considered good markers of early and late glial differentiation, respectively.[30] The C6 glioma cell line is classified as an astrocytoma. The 9L cell line is a gliosarcoma, a rare glioblastoma variant, that exhibits a genetic profile similar to that of primary (de novo) glioblastomas.[31] U87 and U373 cell lines derive from highly invasive grades III and IV human tumors. Primary rat cultured astrocytes expressed high GFAP and low vimentin levels (Fig. 1A and B). Vimentin and GFAP showed different expression levels among the 4 glioma cell lines. GFAP was strongly expressed in C6 and U373, while it was weakly expressed in 9L and almost absent in U87, as evident in both RT-PCR (Fig. 1A) and western blotting analysis (Fig. 1B). In contrast, vimentin levels were strong in 9L, U87, and U373, while C6 exhibited levels comparable to those observed in primary astrocytes (Fig. 1B). Thus, low vimentin/high GFAP levels appear to be a feature of more differentiated cells such as C6, whereas high vimentin/low to absent GFAP levels are typical of less differentiated cells such as 9L and U87. U373 exhibited high levels of both vimentin and GFAP.

Differential Expression of Notch Receptors Is Associated With Expression of Glial Differentiation Markers in Glioma Cell Lines

The expression profile of Notch receptors in glioma cell lines and in primary astroglial cultures was studied by RT-PCR (see Table 2 for primer sequences) and western blot analysis. C6 expressed Notch1, -2, and -4 mRNAs (Fig. 1C), but only Notch1 protein was strongly expressed, whereas Notch2 and Notch4 protein levels were low and Notch3 almost absent (Fig. 1D). Overall, C6 showed a similar pattern of Notch expression as did primary rat astrocytes, which expressed the mRNAs for all 4 Notch receptors (Fig. 1C) but only Notch1 at protein level (Fig. 1D). In contrast, 9L, which also expressed Notch1, -2, and -4 mRNAs (Fig. 1C), exhibited a different pattern of Notch protein expression than C6 and astrocytes, with low Notch1, high Notch2, low to absent Notch3, and moderate to high levels of Notch4 (Fig. 1D). The expression pattern of Notch receptor proteins in U87 resembled that of 9L with low or undetectable Notch1, low levels of Notch3, and moderate to high Notch2 and Notch4 (Fig. 1D). U373 expressed all Notch receptors, with the highest levels for Notch1 and -2 (Fig. 1D). Interestingly, a precise correspondence between the expression of mRNAs and

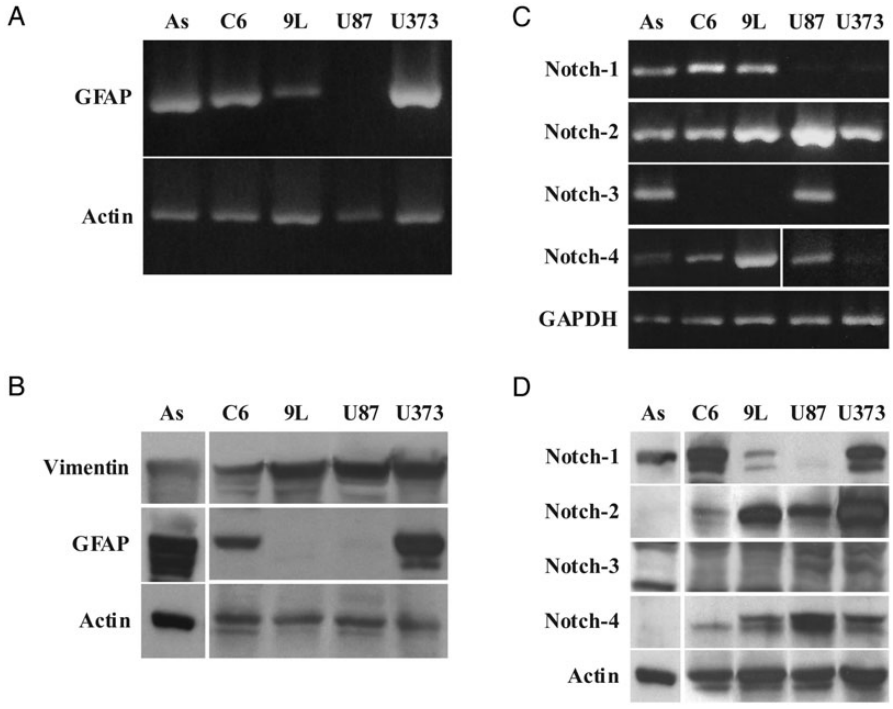

Fig. 1. Vimentin and GFAP are differentially expressed in rat primary astroglial cell culture (As) and C6, 9L, U87, and U373 glioma cell lines as assessed by RT-PCR (A) and western blot analysis (B). Differential Notch mRNA expression levels were detected by RT-PCR (C) in As and C6, 9L, U87, and U373 glioma cell lines. GAPDH was analyzed as a positive control of mRNA expression. Notch4 mRNA expression levels were analyzed by using 2 diverse primer pairs as indicated in Table 2. One primer pair was specific for the rat and the second one for human Notch4 (see Table 2). (D) Representative western blot showing Notch1, -2, -3, and -4 expression levels in As and C6, 9L, U87, and U373 glioma cell lines. One hundred micrograms of proteins were loaded in each lane. Experiments were repeated 3 times with similar results.

protein levels was not always found. For example, while U87 and U373 exhibited similar low levels of Notch1 mRNA, they exhibited different levels of Notch1 protein, which was almost undetectable in U87 and strong in U373 (Fig. 1C and D). In contrast, cultured astrocytes exhibited moderate levels of Notch2 and Notch3 mRNAs but no levels of the corresponding proteins (Fig. 1C and D).

Overall these data suggest that high expression of Notch1/low expression of Notch 4 and vice-versa are associated with more and less differentiated cell lines, respectively.

Notch Receptor Expression in Human Glioma Biopsies

To examine whether a differential expression of Notch1–4 is also found in human pathology, the expression of Notch1 and Notch4 was analyzed in freshly resected human biopsies of low- and high-grade gliomas by western blot analysis (Table 3). Notch1 expression level was moderate in grade II astrocytomas and low or absent in the majority of GBM (Table 3; Fig. 2). Notch4 was differently expressed in grade II astrocytomas (1 absent, 1 moderate, 3 strong) and highly expressed in the majority of GBM (Table 3, Fig. 2). In normal resected tissue Notch1 was low, whereas Notch4 was absent (Table 3). Furthermore, to investigate Notch signaling, we examined the expression of Hes1, a gene activated downstream of Notch signaling. Interestingly, Hes1 expression levels were low in grade II glioma samples, but variable in GBM (Fig. 2 and Table 3). The percentage of Ki-67–positive cells for each sample was calculated as an indicator of proliferation (Table 3).

The expression of the Notch receptors was further evaluated through immunohistochemistry in biopsies of primary glial tumors of different WHO grades (Fig. 3). Normal white matter biopsies did not show any detectable immunolabeling for the 4 Notch receptors (Fig. 3A, B, C, D, A^1, B^1, C^1, D^1), while differences in the expression levels of the 4 Notch receptors were observed in glioma biopsies of different grades.

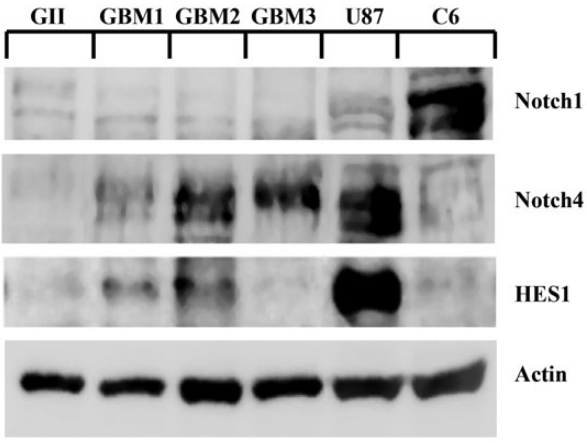

Fig. 2. Representative western blot showing Notch1, Notch4, and Hes1 expression levels in homogenates of human glioma biopsies (GII = low-grade glioma; GBM1, GBM2, GBM3 = high-grade gliomas). U87 and C6 glioma cell lines were included as control for the expression levels of Notch1 and Notch4. Fifty micrograms of proteins were loaded in each lane.

As shown in Fig. 3, a strong nuclear and cytoplasmic immunoreactivity for Notch1 was observed in grade II astrocytoma (Fig. 3E and F) and in anaplastic astrocytoma (Fig. 3K and L). Notch1 immunoreactivity was variable and cytoplasmic in GBM (Fig. 3Q and R). Furthermore, the evaluation of labeled tissue sections confirmed that Notch1 had a high score, in terms of intensity and frequency, in astrocytoma and in anaplastic astrocytoma that decreased in glioblastoma (Fig. $3A^1$). Weak cytoplasmic immunoreactivity for Notch2 was observed in grade II astrocytoma (Fig. 3G). Notch2 immunoreactivity showed increasing level and density in anaplastic astrocytoma (Fig. 3M), to become strong in cytoplasm of GBM cells (Fig. 3S, and T). These results match perfectly with the evaluated score (Fig. $3B^1$). Notch3 (Fig. 3C, H, N, U, and C^1) showed a nuclear and cytoplasmic immunoreactivity in grade II astrocytoma (Fig. 3H), cytoplasmic immunoreactivity in anaplastic astrocytoma (Fig. 3N), and a stronger cytoplasmic immunoreactivity in GBM (Fig. 3U). Notch4 showed strong nuclear and cytoplasmic immunoreactivity in grade II astrocytoma (Fig. 3I and J). The labeling intensity of Notch4 increased from anaplastic astrocytoma (Fig. 3O and P) to GBM (Fig. 3V and Z). The frequency and intensity of Notch4 increased from astrocytomas to GBM (Fig. $3D^1$).

Notch Receptor Expression in Primary Human Glioma Cell Cultures

Human primary glioma cell cultures obtained from a high-grade GBM tumor (FL75; Table 3) were grown in the presence of 10% FCS, 10% FCS and then shifted to NSF, and NSF directly (see Material and Methods and Fig. 4A). While cells in the presence of FCS grew as a monolayer of flat cells attached to the petri dish, cells either directly grown in NSF or shifted to NSF formed aggregates of cells resembling neurospheres or detached from the bottom of the dish and started to grow as neurosphere-like aggregates (tumorspheres).

The expressions of stemness markers, CD133 and nestin, and glial differentiation markers such as vimentin and GFAP were analyzed in GBM cell cultures grown in these different conditions and compared with those found in biopsies by western blot (Fig. 4B) and immunocytochemistry/confocal microscopy (Fig. 4C). CD133 and nestin expressions were high and moderate, respectively, in FCS and low in tumorspheres (Fig. 4B and C). Vimentin expression was high in FCS to slightly decreased in NSF medium (Fig. 4B and C). GFAP showed low levels in any culture condition (Fig. 4B and C), while it had a high expression level in the biopsy. This result might be ascribed to the presence of reactive astrocytes in the biopsies.

Notch1 levels were low in cells grown in the presence of FCS and low or absent in cells grown in NSF as tumorspheres (Fig. 4B). In contrast, Notch4 levels were moderate to high in FCS and dramatically reduced when cells were either shifted to or grown directly in NSF medium (Fig. 4B). Confocal analysis in FCS revealed that Notch1 was mainly cytoplasmic, whereas Notch4 distribution was more nuclear than cytoplasmic. In tumorspheres, Notch1 was mostly undetectable, whereas Notch4 was detected in few cells and was more cytoplasmic than in FCS (Fig. 4C).

Levels of Notch1 and Notch4 were further examined by western blot analysis in glioma cell cultures from different WHO grades either grown as monolayers in FCS or shifted to NSF medium. Primary cultures obtained from both low (grade II)- and high

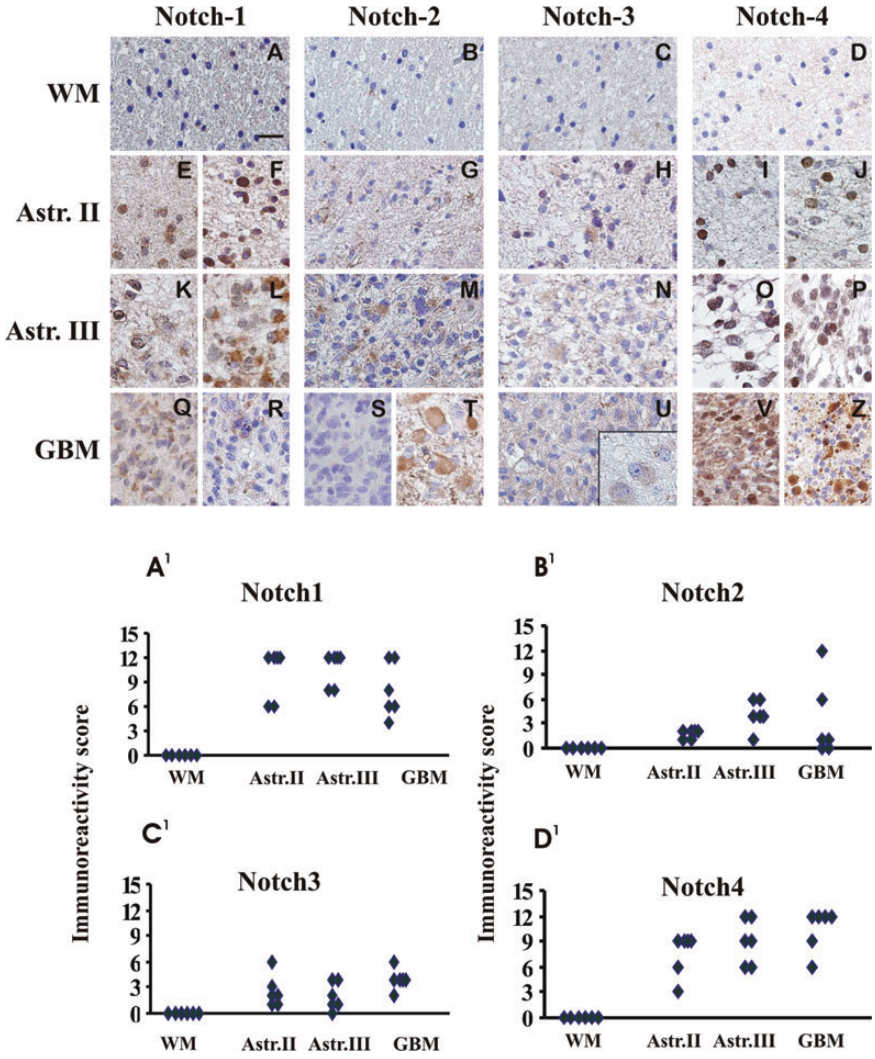

Fig. 3. Expression of Notch1,-2, -3, -4 immunoreactivity (IR) in human gliomas. Notch1 IR panels: A, E, F, K, L, Q, and R; Notch2 IR panels: B, G, M, S and T; Notch3 IR panels: C, H, N, U; Notch4 IR panels: D, I, J, O, P, V, Z. Panels: A, B, C, D normal white matter (WM), showing no detectable glial Notch1,-2, -3, -4 labeling. Panels E, F: representative photomicrographs of Notch1 IR in astrocytoma II (Astr. II), with strong nuclear and cytoplasmic Notch1 staining. Panels K, L: representative photomicrographs of Notch1 IR in anaplastic astrocytoma (Astr. III). Panels Q, R: representative photomicrographs of Notch1 IR in GBM, displaying variable IR and cytoplasmic staining. Panel G: representative photomicrographs of Astr. II, showing weak Notch2 staining. Panel M: Notch2 IR in Astr. III with cytoplasmic labeling. Panels S, T: representative photomicrographs of Notch2 IR in GBM, displaying variable IR and cytoplasmic staining. Panel H: representative photomicrographs of Astr. II, showing weak Notch3 staining. Panel N: Notch3 IR in Astr. III. Panel U: representative photomicrographs of GBM showing weak cytoplasmic staining. Panels I, J: representative photomicrographs of Notch4 IR in Astr. II, showing strong nuclear staining. Panels O, P: representative photomicrographs of Notch4 IR in Astr. III. Panels V, Z: representative photomicrographs of Notch4 IR in GBM displaying variable IR and both nuclear and cytoplasmic staining. Scale bar in A: 100 μm; B, C, D, and G: 100 μm; E, F, H–Z: 50 μm. Panels: A^1, B^1, C^1, and D^1 *scatter plots* showing the distribution of Notch1–4 IR scores (total score; see for details Method's section) in control WM and in astrocytic tumors: Astr. II, Astr. III, and GBM.

(GBM)-grade gliomas grown in the presence of FCS showed low/moderate levels of Notch1. Notch1 levels were not substantially changed when cultures from both low- and high-grade tumors grown in FCS were shifted to NSF (Fig. 5). In contrast, Notch4 expression level was low in grade II astrocytoma primary FCS cultures and very high in GBM primary FCS cultures (Fig. 5). Furthermore, cultures obtained from both low- and high-grade tumors grown in FCS and shifted to NSF medium showed a drastic reduction of Notch4 expression levels (Fig. 5). Hes1 expression levels were similar in primary GBM cultures grown as monolayers or as neurospheres/tumorspheres, whilst an upregulation of Hes1 was observed in grade II primary cultures grown as neurospheres/tumorspheres (Fig. 5).

Gamma-Secretase Inhibitor Treatment Reduces Cell Viability of Glioma Cell Lines

In order to study the possible involvement of Notch signaling in glioma cellular proliferation and/or survival, different concentrations of GSI were added to glioma cell lines. Cells were grown in the presence of FCS or shifted to NSF as described above and cell viability was assessed 24 h after the exposure to GSI by MTT

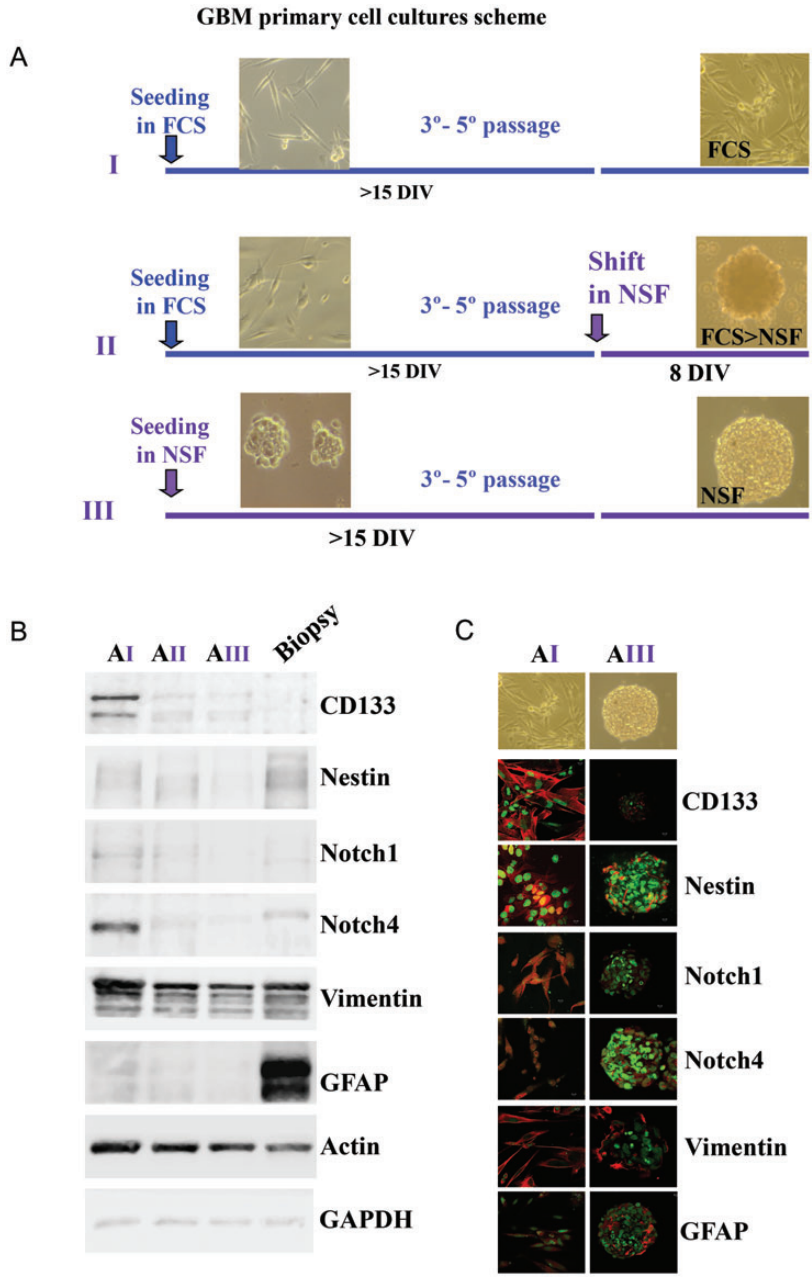

Fig. 4. (A) Scheme showing the experimental procedure used to obtain primary cultures from GBM**. **I** Cells were seeded and grown in the presence of 10% FCS; **II** cells were seeded and grown in 10% FCS and then shifted to NSF medium when 80%–90% confluent; **III** cells were seeded directly and grown in a serum-free medium with growth factors (NSF). ** GBM cultures were prepared from FL75 listed in Table 3. Images are representative of primary cultures in the conditions already indicated. (B) Western blot analysis showing CD133, nestin, Notch-1 and Notch-4, vimentin, and GFAP expression levels in primary human glioma cell cultures under the different growth conditions listed (AI, AII, and AIII). Last lane is loaded with total proteins obtained from the tissue homogenate (part of the biopsy from where primary cultures were obtained). Actin and GAPDH expression levels are shown as controls of equal amount of total proteins loaded per lane. (C) Confocal images of immunocytochemistry experiments carried out in primary GBM cultures under different growth conditions by using the same antibodies as in B (**AI** seeded and grown in FCS and **AIII** seeded and grown in NSF).

assay. As observed in human cultured gliomas, the expression of Notch4 was reduced in both C6 and U87 cell lines when cultures grown in FCS were shifted to NSF medium (Fig. 6). The reduction of Notch4 was robust in U87 cell lines, which exhibit a strong expression of Notch4 in FCS. Notch1, which does not undergo significant changes in human cultured gliomas (Fig. 5), was reduced in C6, where it was highly expressed in FCS, by shifting the medium to NSF (Fig. 6A). Treatment with GSI (10 μM) was efficacious, as revealed by reduction of the intracytoplasmic domain of Notch1 (NICD) in both C6 and U87 glioma cell lines (Fig. 6B).

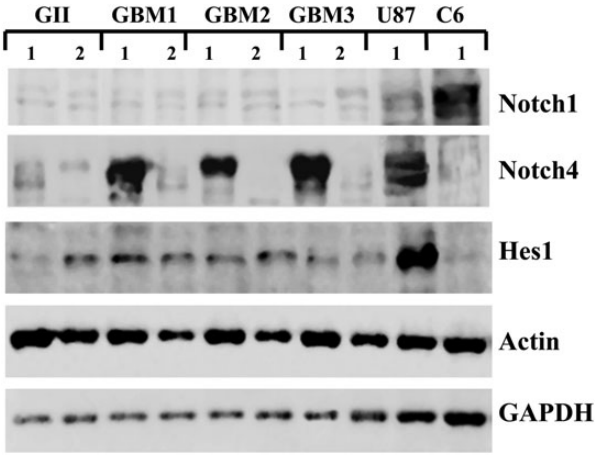

Fig. 5. Representative western blots showing Notch1, Notch4, and Hes1 expression levels in human primary glioma cell cultures (GII = low-grade glioma cultures; GBM1, GBM2, GBM3 = high-grade/glioblastoma cultures. Cultures were obtained from subjects indicated with * in Table 3). U87 and C6 glioma cell lines were included as a reference for the expression levels of Notch1 and Notch4. **1** = total proteins obtained from human primary glioma culture seeded and grown in FCS; **2** = total proteins obtained from human primary glioma culture grown in FCS and shifted to NSF. Actin and GAPDH expression levels are shown as controls of equal amount of total proteins loaded per lane. Twenty-five micrograms of total proteins from glioma cell cultures and 50 μg of total proteins from U87 and C6 were loaded.

Exposure to 10 micromolar GSI for 24 h increased caspase-3 activation in U87 (+135%) and 9L (+60%), an effect that was inhibited by treatment with the caspase-3 inhibitor Z-VAD-FMK (50 μM) (Fig. 6C).

Dose response curves showed that GSI reduced the growth/survival of glioma cell lines with a stronger effect when cells were grown in the presence of serum (9L > U87 > C6, U373). Two-way ANOVA revealed a statistically significant difference among the GSI doses in all cell lines ($P < .001$), indicating that GSI treatment was efficacious under each growth condition. The difference between FCS and NFS was also significant in all cell lines ($P < .001$ for 9L, U87, and U373 and $P < .05$ for C6). Interestingly, the sensitivity of glioma cell cultures to GSI treatment when shifted to NFS was reduced in C6, U87, and 9L, whereas it was higher in U373 (Fig. 6D).

Differential Sensitivity to GSI of Human Glioma Primary Cultures

Cell survival after GSI treatment was also studied in 3 low-grade (grade II astrocytoma) and 8 high-grade (GBM) human glioma cultures, which were grown in FCS or shifted to NSF, as described. All 3 low-grade glioma cell cultures behaved similarly, exhibiting a low sensitivity to GSI only when grown in FCS, whereas they were insensitive when shifted to NSF (Fig. 7A). A high variability was observed in response to GSI treatment among high-grade glioma cultures. Three of 8 GBM cultures behaved similarly to grade II astrocytoma, being moderately sensitive to GSI only in FCS (Fig. 7B), 2 showed equal moderate sensitivity to GSI in both culture conditions (Fig. 7C), while a third group of 2 GBM showed higher sensitivity to GSI when shifted to NSF (Fig. 7D). One GBM was insensitive under both culture conditions (data not shown).

Discussion

Numerous data published during the last decade show the relevant role of Notch signaling as a key component during cell-fate specification in the development of the mammalian nervous system.[6] Notch signaling activates diverse genes, which in turn can activate or inhibit many different cellular functions. Despite the enormous amount of published data on Notch in brain development and human diseases, the expression and the role of the 4 Notch receptors in neoplasms, such as gliomas, have not been investigated systematically.

Our study analyzes the differential expression of the 4 diverse Notch receptors in glioma cell lines, in human biopsies of gliomas of different WHO grades, and in human primary glioma cell cultures obtained from freshly resected gliomas of different WHO grades. Furthermore, the possible involvement of Notch signal transduction in tumoral cellular survival/proliferation was also studied. Overall, our data suggest that higher expression of Notch1 is indicative of higher differentiation, whereas high expression of Notch4 may indicate a lower differentiation grade. This indication was firstly drawn from results obtained in cell lines and corroborated from those obtained in human biopsies. We have found that the expression of the Notch receptors correlates with that of vimentin and GFAP in cell lines. In fact, cells exhibiting high levels of GFAP, such as C6 and U373, also expressed high levels of Notch1, while cells expressing low levels of GFAP, such as 9L and U87, had low levels of Notch1 protein. In contrast, high vimentin levels corresponded to both high Notch2 and Notch4, as in U373. Accordingly, Notch1 expression level was moderate in glioma biopsies from low-grade tumors and maintained in high-grade ones, even with different expression levels, while Notch4 expression level was variable in low-grade glioma biopsies, with the major expression in high-grade ones. Moreover, the analysis of the biopsies by immunostaining for the 4 Notch receptors has highlighted that even though Notch1 is present in grade IV/GBM gliomas, it has a minor frequency and intensity compared with Notch4. These data were further strengthened from results obtained in human primary cultures where we observed a low/moderate expression of Notch1 in low-grade tumor (grade II astrocytoma) cultures and in cultures from high-grade tumors, while Notch4 expression was low in grade II and very high in cultures from high-grade gliomas (GBM). Interestingly, when primary cultures grown in the presence of 10% FCS were shifted to a serum-free medium plus growth factors (see Material and Methods section) the Notch1 expression levels were not apparently affected, while a dramatic decrease was evident in Notch4 expression levels. Moreover, GBM cultures grown in the presence of serum expressed higher levels of CD133 and nestin proteins than GBM cultures grown in the absence of serum as neurospheres. These results show that cancer cells grown in the presence of serum exhibit molecular signature of less differentiated cells compared with neurospheres. The dramatic reduction of Notch4 expression might be associated with a tentative differentiation of the majority of cells contained in the tumorspheres and may further substantiate our conclusion that high Notch4 expression is associated with a less differentiated and possibly more aggressive tumor. Interestingly, a robust

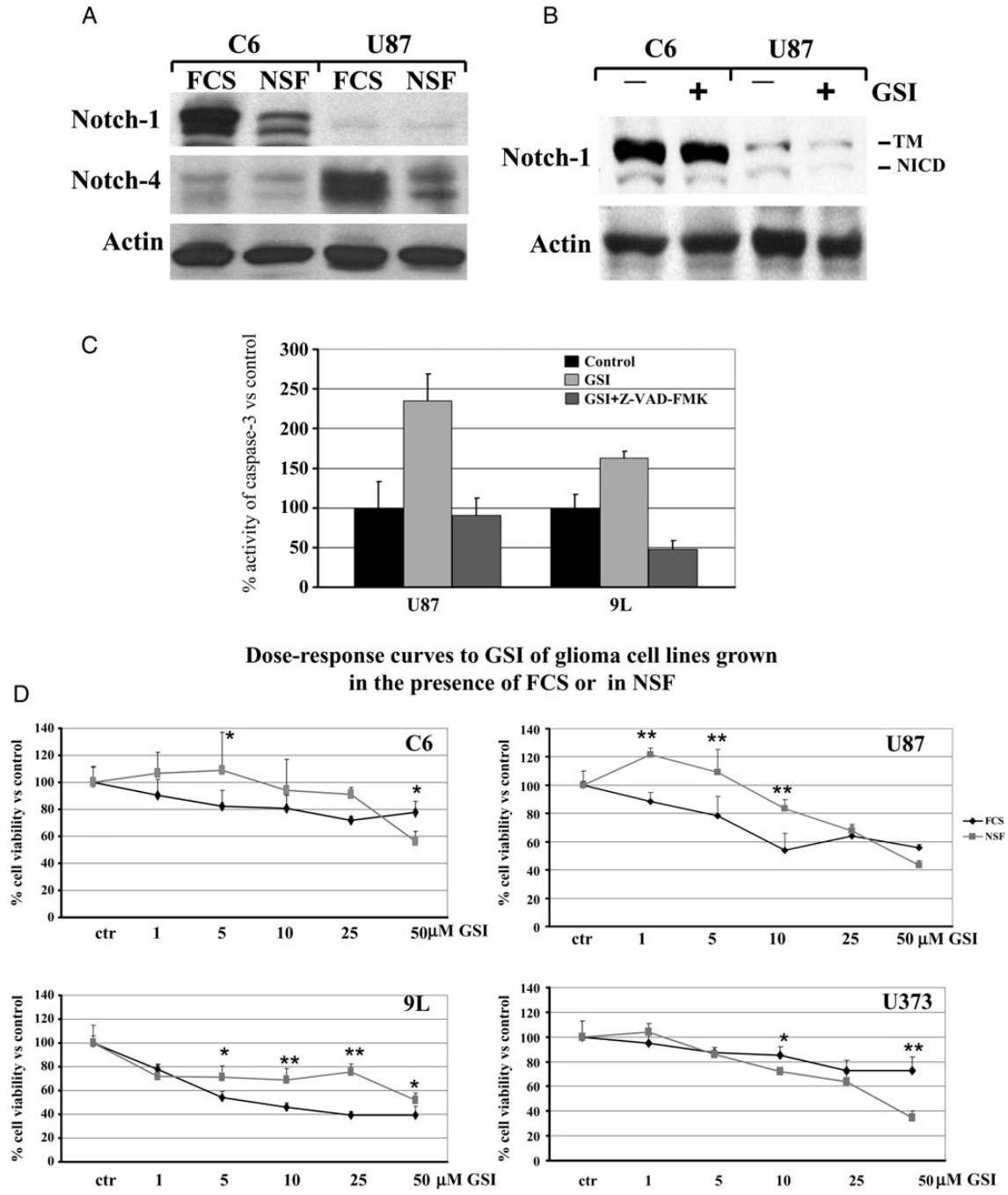

Fig. 6. Analysis of 24 h GSI treatment in glioma cell lines. (A) Western blot analysis showing Notch1 and Notch4 expression levels in C6 and U87 grown in the presence of 10% FCS or in NSF. (B) Western blot analysis showing basal levels of Notch1 expression and reduction of NICD after 24 h treatment with 10 μM GSI of C6 and U87 grown in 10% FCS. Actin expression levels are shown as control of equal amount of total protein loaded per lane (100 μg). (C) Histogram showing caspase-3 activity in U87 and 9L glioma cell lines treated with GSI alone or in the presence of Z-VAD-FMK, a pan-caspase inhibitor. Values are the mean of 4 different samples, and error bars represent standard deviation. (D) Dose-response curves obtained after 24 h treatment with 1, 5, 10, 25, and 50 μM GSI of glioma cell lines grown in FCS or in NSF. Two-way ANOVA revealed a statistically significant difference between FCS and NSF growth conditions in all glioma cell lines (*$P < .05$, ** $P < .001$ different between FCS and NSF growth condition by pairwise multiple comparison procedure within each GSI concentration, Holm-Sidak method).

reduction of Notch4 has been detected in neurospheres undergoing differentiation, suggesting again that a higher Notch4 expression might be associated with a less differentiated state.[32]

Several papers have recently reported a different expression of Notch1 and Notch2 in human gliomas, with different results and conclusions regarding tumor progression and prognosis. Notch1

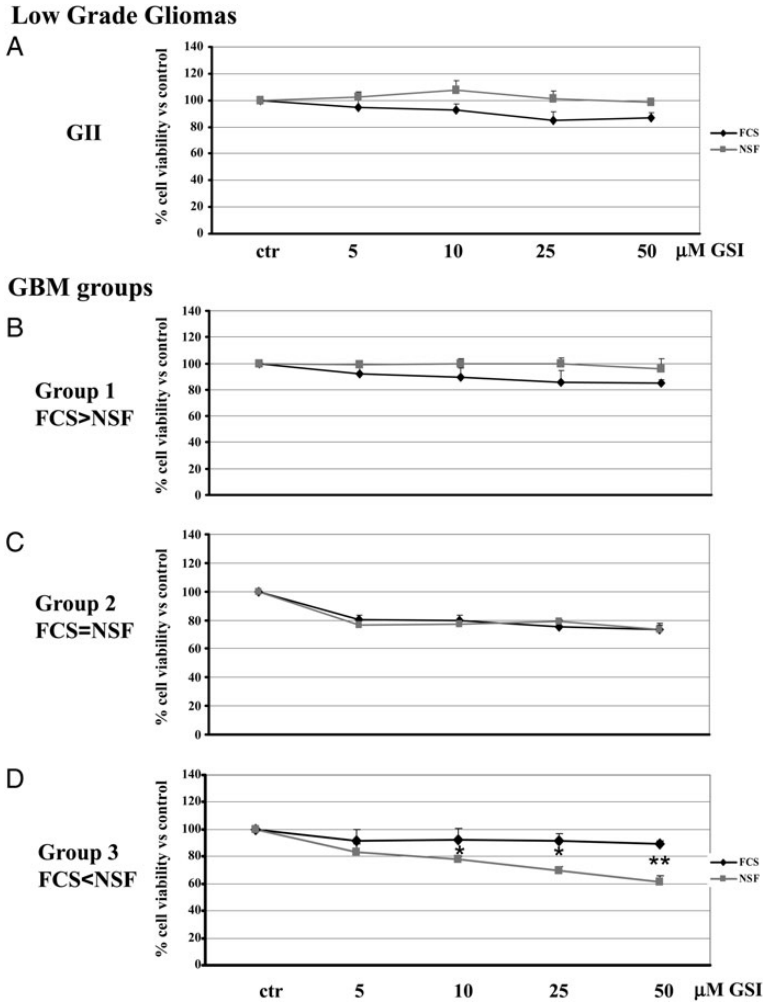

Fig. 7. Dose-response curves obtained after 24 h treatment with 1, 5, 10, 25, and 50 μM GSI of primary human glioma cell cultures grown in FCS or grown in FCS and shifted to NSF. GII = low-grade glioma cultures; GBM groups = high-grade/glioblastoma cultures. In **A** low-grade gliomas GII the graph is the result of 3 diverse GII cultures; in **B group 1** of high-grade/glioblastomas the graph is the result of 3 diverse GBM cultures. FCS cultures are more sensitive to GSI than NSF cultures. In **C group 2** of high-grade/glioblastomas the graph is the result of 2 diverse GBM cultures. FCS and NSF cultures are equally sensitive to GSI. In **D group 3** of high-grade/glioblastomas the graph is the result of 2 diverse GBM cultures. FCS cultures are less sensitive to GSI than NSF cultures. All experiments have been repeated 3 times. Two-way ANOVA revealed a statistically significant interaction between FCS and NSF growth conditions and GSI doses in group 3 (*$P < .05$, **$P < .001$ different between FCS and NSF by pairwise multiple comparison procedure within each GSI concentration, Holm-Sidak method).

has been detected to be abnormally expressed in gliomas of all grades but absent in a subset of grade IV gliomas,[33] although no conclusion was drawn regarding behavior of Notch1-negative high-grade gliomas. Purow and colleagues[21] report that Notch1 is higher in gliomas of grades II and III than in glioblastomas. A different paper reports that Notch1 nuclear staining was found to be associated with a better outcome in high-grade glioma subtypes compared with poor prognosis subtypes.[34] Similarly to what we have found in glioblastomas, a high expression of Notch2 and low expression of Notch1 have been detected in medulloblastomas, where cell proliferation, soft agar colony formation, and xenograft growth were all promoted by Notch2 and inhibited by Notch1.[19] Accordingly, loss of Notch2 was detected in a subset of glioma patients with favorable prognosis.[23] In contrast, other data identify Notch1 as an independent prognostic factor in gliomas, with Notch1 increasing levels from grade I to grade IV,[35] whereas Notch2 was undetectable in high-grade gliomas. Furthermore, knocking down Notch1 or enforcing Notch2 overexpression had the effect of suppressing cell growth and invasion as well as inducing apoptosis.[36]

Besides the possible role of the Notch1–4 expression pattern as a marker of glioma malignancy and therefore of clinical outcome, each Notch receptor may play a different role in tumorigenesis. Several papers have highlighted a role for Notch signaling in tumorigenesis, especially in leukemia and solid tumors, but the role of each Notch receptor is far less clear. A recent paper reports that active forms of Notch1, Notch2, and Notch3 have differing abilities to induce glial tumors and that glial progenitors in the optic nerve and retina are particularly susceptible to Notch3-driven transformation compared with those in the rest of the

CNS.[24] While Notch1, -2, and -3 overexpression was not tumorigenic in glial progenitor from the brain, the role of Notch4 is presently not known. Future studies will establish whether Notch4 may be considered a marker of tumor aggressiveness and progression or a significant mediator of tumorigenesis.

The analysis of Hes1 expression, a gene activated downstream of the Notch signal, revealed a variable expression in GBM with no apparent correlation with the expression of Notch1 and Notch4. Hes1 is a known transcriptional repressor involved in the maintenance of neural stem cells through the repression of proneural genes.[37] A similar analysis in GBM primary cultures in different growth conditions indicated a basal expression of Hes1 with no clear difference in cells grown as monolayers or tumorspheres. This might indicate that Hes1 is likely not under the control of Notch4 and cannot be considered a marker of glioma' differentiation. Further investigations are necessary to study expression of genes downstream of Notch4 cleavage.

Since cells grown in the presence of serum exhibited hallmarks of less differentiated cells, we compared the sensitivity of different glioma cell cultures grown under different conditions with increasing concentration of GSI. The results obtained in glioma cell lines suggested that the sensitivity of GSI was reduced in NSF, that is, in a condition in which the expression of Notch was drastically reduced. The same analysis of primary human glioma cell cultures grown in the presence of FCS or in NSF have revealed a more complex picture than the one observed in glioma cell lines. In fact, cultures from low-grade gliomas showed a similar pattern to that observed for C6 (the more differentiated glioma cell lines), while cultures obtained from GBM showed complex responses to GSI treatment in the 2 culture conditions. GSI treatment of GBM primary cultures allowed us to divide the cultures into 3 groups, the first of which showed a behavior toward GSI treatment similar to low-grade cultures, the second showed a similar sensitivity to GSI in both culture conditions, while the third was more sensitive to GSI when cells were kept in NSF. These observations led us to hypothesize that the glioma survival pathway gained by γ-secretase activation with Notch receptor cleavage could not be the only one involved. Moreover, Notch signaling could also be activated in a noncanonical way, so that its inhibition could be bypassed and cells survive despite GSI treatment. However, more experimental work is needed to get clear answers in the attempt to unravel the complex tangle of events important for the survival of cancerous cells.

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