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Abstract. p75^{NTR}, a member of the tumor necrosis factor superfamily, plays a key role in numerous physiological processes, including cell survival or apoptosis. Yet, the associated signaling pathways remain poorly understood. Similar to Notch, γ-secretase cleavage is implicated in the p75^{NTR} signaling pathway leading to nuclear translocation of the intracellular domain and cell death. Fas receptor activation was found to promote cell death apoptosis in several cell lines. The goal of this study was to determine the respective role of p75^{NTR} and Notch in the resistance to Fas-induced apoptosis in the U-87 MG glioblastoma cell line. Using the γ-secretase inhibitor, we investigated the modulation of Fas-induced apoptosis dependent on p75^{NTR}-Fas receptor interaction. Whereas the U-87 MG cells expressed the Fas receptor at the cell membrane, apoptosis induced by Fas activation was decreased by the γ-secretase inhibitor. These data suggest that γ-secretase is implicated in p75^{NTR} and Fas interaction leading to cell death signaling.

Introduction

Glioblastoma, a brain tumor that develops from glial cells, mostly in adults, is considered to be the most malignant form of gliomas, which represent half of all primitive brain tumors. Its poor prognosis is related to the high malignant status and the absence of effective therapies, explained by their intrinsic resistance to many cytotoxic agents (1,2). As the development of new therapeutic strategies is of crucial importance, the mechanisms underlying the resistance to apoptosis induction need to be elucidated in order to define potential strategies involving therapeutic association of molecules, such as those related to Fas-mediated apoptosis. The Fas receptor, a member of the tumor-necrosis factor receptor superfamily, mediates apoptosis in certain sensitive cells when it is activated by Fas ligand (FasL). Therefore, it has been implicated in tumor regression, such as in glioma, especially when used in combination with other therapeutic molecules, such as etoposide and dexamethasone as reported in an experimental model of glioblastoma in the nude rat (3). However, potential residual tumor cells may reduce survival depending on fine interactions that counterbalance the Fas-signaling pathway. Accordingly, in another malignant cell line, a neuroblastoma line, we reported that the Fas receptor interacts with the p75^{NTR} receptor inhibiting Fas-induced apoptosis (4). This resistance to cell death was effective through co-activation of p75^{NTR}, another cell death receptor for neurotrophins, and the Fas receptor, by their natural ligands, BDNF and FasL, respectively, allowing that the Fas and p75^{NTR} pathways are interactive (4). This dual function of p75^{NTR} in apoptosis induction or cell growth and survival is in part due to its transcriptional function resulting from sequential α and γ-secretase actions that cleave p75^{NTR} in the 24-kDa C-terminal fragment and a 19-kDa intracellular domain (ICD) fragment. ICD is translocated into the nucleus (5), where it modulates transcriptional events (6). Recent studies have demonstrated that p75^{NTR} is a critical regulator of glioma invasion (7) endogenously expressed in patient tumors (8). Its biological function in invasion and tumor growth is related to its proteolytic processing by γ-secretase, leading to invasiveness and cell migration and their suppression by γ-secretase inhibitors (8). In addition, γ-secretase inhibitors were found to increase radiosensitivity of glioblastoma, and induce reduction in CD133^{+}-related stem cells (9). As proteolysis through γ-secretase activity is a highly conserved mechanism responsible for intramembrane cleavage of several proteins, such as β-amyloid precursor protein, ErbB4, CD44 and Notch receptor (10), it was speculated by Lin *et al* that γ-secretase inhibitors inhibit CD133^{+} glioblastoma cell lines through inhibition of the Notch signaling pathway (9).

Notch signaling plays a key role in the normal development of many tissues and cell types, through effects on differentiation, survival and/or proliferation that are highly dependent on signal strength and cellular context (11). Perturbations in the regulation of cellular homeostasis trigger malignant transformation. Moreover, Notch1 signaling potentially contributes to cancer development in several different pathways by

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activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin, or by acting on Myc transcription (12).

Accumulating data indicate that deregulated Notch or p75^{NTR} activities may be involved in human cancer, such as glioblastoma (8). In order to improve glioblastoma therapy and patient care, diagnostic and prognostic factors are required.

The goal of the present study was to determine the respective role of p75^{NTR} and Notch in the resistance to Fas-induced apoptosis in the U-87 MG glioblastoma cell line. Using the γ-secretase inhibitor, we searched for a modulation of Fas-induced apoptosis dependent on p75^{NTR}-Fas receptor interaction.

We reported herein that the γ-secretase inhibitor diminished apoptosis through Fas activation and we hypothesized that the suppression of the proteolysis of p75^{NTR} interferes with the Fas-p75^{NTR} recruitment of apoptosis.

Materials and methods

Cell line culture and treatments. U-87 MG human glioblastoma cells (ATCC, Manassas, VA, USA) were cultured in MEM with Earl's salts (Invitrogen) supplemented with 10% decomplemented foetal calf serum (FCS; Seromed, Invitrogen), 1.5 g/l sodium bicarbonate, 1% non-essential amino acids, sodium pyruvate (1 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM) and fungizone (0.1%) (Invitrogen). Cells were grown in 25-cm^2 flasks (Nunc, France) at 37˚C in a humidified 5% CO_2-95% air incubator. At subconfluence, cells were always recovered with versene (Invitrogen) and cultured in Lab-Tek chamber slides (Nunc) at 10^4 cells/well or in 6-well plates (Nunc) at 10^5 cells/well for 48 h and were treated for 24 h with 7C11, an agonistic anti-Fas monoclonal antibody (mAb) at 100 ng/ml (Beckman Coulter Inc., Fullerton, CA, USA), the γ-secretase inhibitor at 1 μM (Calbiochem, France) or both molecules.

Flow cytometry. U-87 MG cells were cultured in 6-well plates (10^5 cells/well) at basal state or stimulated by agonistic anti-Fas mAb or a γ-secretase inhibitor or both molecules during 24 h. U-87 MG cells were first released with versene permeabilized or not using Triton X-100 (0.1%; Promega, France) and resuspended in 10% FCS/90% versene with either rabbit anti-Notch1 (C-20 or H131, 1/100; Santa Cruz Biotechnology), anti-Notch2 (25-255, 1/100; Santa Cruz Biotechnology), anti-Delta1 (H-265, 1/100; Santa Cruz Biotechnology), anti-Delta4 (H-70, 1/100; Santa Cruz Biotechnology), anti-Jagged1 (H-114, 1/100; Santa Cruz Biotechnology) or anti-Jagged2 (H-143, 1/100; Santa Cruz Biotechnology) polyclonal antibodies (Abs). Two anti-Fas mAb phycoerythrin (PE)-conjugated recognizing the N- or C-terminus domain (UB2, 1/5; Immunotech, Marseille, France; or B-10, 1/50; Santa Cruz Biotechnology) were used. p75^{NTR} expression was studied with an anti-p75^{NTR} polyclonal Abs (H-137, 1/20; Santa Cruz Biotechnology) directed against the extracellular domain of p75^{NTR}. Immature cell marker, an anti-vimentin mAb (1/50; Santa Cruz Biotechnology) was also evaluated. Controls were performed with rabbit irrelevant Ig (Santa Cruz Biotechnology) or mouse irrelevant Ig as isotypic controls. After a 30-min incubation on ice, non-conjugated-rabbit Abs were detected with a swine anti-rabbit Ig fluorescein isothiocyanate (FITC)-conjugated antibody (Dako-Cytomation, Trappes, France) diluted at 1/100, in 10% FCS in versene during 30 min on ice. Finally, cells were resuspended in 10% FCS/90% versene. Each analysis was performed on at least 10,000 cells and repeated three times. Relative fluorescence intensity was measured by fluorescence-activated cell sorter analysis (Cytomics FC200; Beckman Coulter and LSR Fortessa, BD).

Assessment of in vitro apoptosis induction. To determine the apoptotic effect of Fas activation, cells were stimulated for 24 h with 7C11 mAb or a γ-secretase inhibitor alone or with both molecules. Two methods were used: i) Soluble cytoplasmic nucleosome detection. After a 24-h treatment of cells as described above, the rate of apoptosis was measured using cell death detection ELISA Plus (Roche Diagnostic, Meylan, France) according to the manufacturer's instructions. ii) Annexin-V staining. After two washes in PBS, cells were incubated with Annexin V-FITC (Beckman Coulter) solution (a marker of phosphatidylserine exposure to the cell membrane during early apoptotic process) containing propidium iodide (a DNA-intercalating dye to detect necrotic cells) for 15 min on ice. Cells were washed according to the manufacturer's instructions and analysis was directly performed by flow cytometry (Cytomics FC200; Beckman Coulter).

Cell cycle analysis. After a 24-h stimulation with 7C11, cell cycle analysis was performed using propidium iodide cell incorporation during 15 min and flow cytometric analysis (Cytomics FC200).

Statistical analyses. Each assay was performed at least in triplicate. For direct comparison of the apoptosis level according to the different conditions of treatment (Basal, 7C11, γ-secretase inhibitor and 7C11 + γ-secretase inhibitor), statistical significance was determined by one-way analysis of variance with Statview 5.0 software. A value of $p<0.05$ was considered to denote statistical significance.

Results

Fas and p75^{NTR} membrane expression in the U-87 MG cell line. Flow cytometry was performed using anti-Fas and p75^{NTR} extracellular domain antibodies. As expected, Fas was detected at the membrane level (3) with ~100% of Fas-expressing cells (Fig. 1A). To determine that the Fas receptor was the full length receptor, potentially functional, and not a truncated form of Fas, staining was performed using specific mAb directed against anti-intracellular domain of Fas, recognizing the death domain. Data showed similar patterns of staining to those obtained with extracellular anti-Fas mAb, assessing that Fas expressed in U-87 MG cells deals with the full-length Fas protein containing the death domain (Fig. 1B).

In parallel, we searched for p75^{NTR} expression in U-87 MG cells. By contrast, by flow cytometry only 15% of cells expressed p75^{NTR} at the membrane level, while 70% was detected after cell permeabilization (Fig. 1C and D), as previously described (13). In order to search for potential interactions with the Notch pathway during Fas activation, we studied Notch expression in U-87 MG cells.

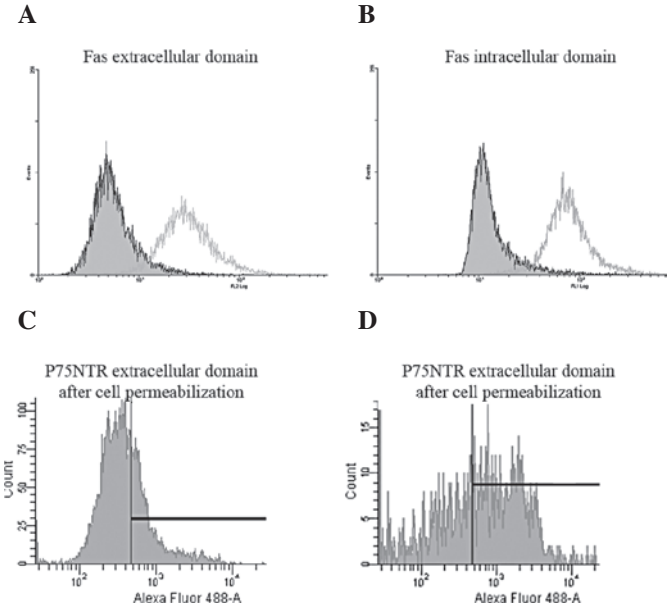

Figure 1. Detection of Fas and $p75^{NTR}$ by flow cytometry in the U-87 MG cell line. (A) Extracellular domain of the Fas receptor was detected in 100% of U-87 MG cells at the membrane level using phycoerythrin-conjugated antibody (UB2 clone). (B) Intracellular domain of the Fas receptor was detected in 100% of U-87 MG cells after cell permeabilization using phycoerythrin-conjugated antibody (B-10 clone). (C) Extracellular domain of $p75^{NTR}$ was detected only in 15% of the U-87 MG cells at the membrane level using polyclonal antibody recognizing the extracellular domain of $p75^{NTR}$ revealed by anti-rabbit fluorescein isothiocyanate-conjugated antibody. (D) Extracellular domain of $p75^{NTR}$ was detected in 80% of U-87 MG cells after cell permeabilization.

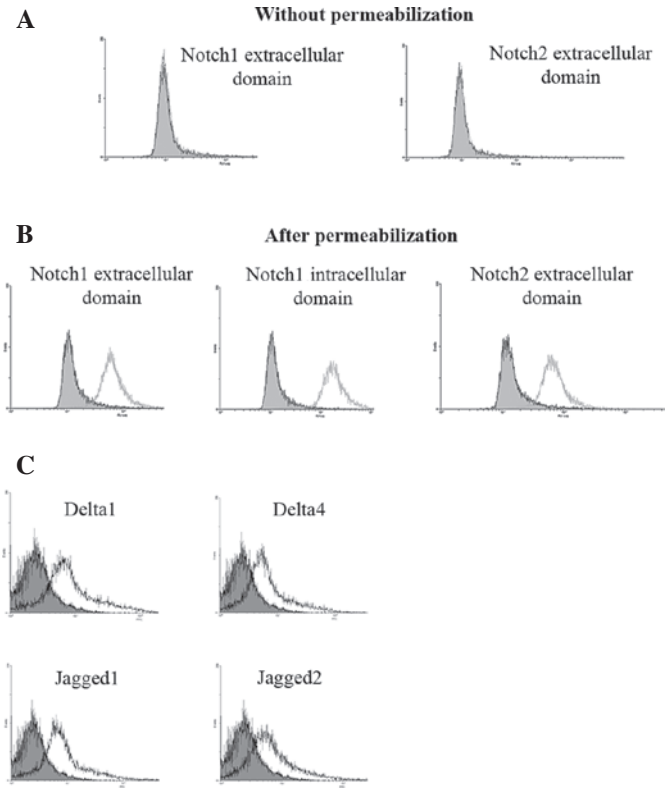

Figure 2. Detection of Notch1, 2 and their ligands by flow cytometry in the U-87 MG cell line. (A) Neither Notch1 nor Notch2 were detected by flow cytometry without cell permeabilization in U-87 MG cells. (B) Notch1 and 2 were sequestered in the cytoplasm, and only detected after cell permeabilization of U-87 MG cells. (C) Delta 1, Delta 4, Jagged 1 and Jagged 2 were only detected after cell permeabilization. These Notch ligands were sequestered in the U-87 MG cell cytoplasm.

Notch1, 2 and their ligands are sequestered in the U-87 MG cells. Using specific antibodies directed against extracellular domains of Notch by flow cytometry (Fig. 2) or immunocytochemical (data not shown) methods, we did not detect Notch1 as well as Notch2 expression on the cell membrane (Fig. 2A). By contrast, Notch1 and Notch2 proteins were only detected after cell permeabilization in U-87 MG cells. Their detection with extracellular- and intracellular-specific anti-Notch Abs in U-87 MG permeabilized cells confirmed that their full-length proteins were sequestered into the cytoplasm (Fig. 2B). In addition, we confirmed by flow cytometry that Notch ligands Delta 1 and 4 and Jagged 1 and 2 were also expressed in intracellular compartments of U-87 MG cells (Fig. 2C).

Fas activation induces apoptosis without modifying cell cycle, vimentin or Notch expression in the U-87 MG cell line. After a 24-h incubation with the agonistic anti-Fas mAb 7C11 (100 ng/ml), only 15% of cells were detected as apoptotic expressing cleaved caspase-3, assessing that U-87 MG cells are relatively resistant to apoptosis (Fig. 3A). In addition, using the same experimental conditions, no variation in cell cycle was observed (Fig. 3B). Vimentin, an immature cell marker, was detected in U-87 MG cells (14) by flow cytometry without change of its expression after anti-Fas 7C11 treatment (Fig. 3C).

Furthermore, Notch1 expression, the most implicated receptor of the Notch family in glioblastoma was studied in Fas-activating conditions in U-87 MG cells treated with 7C11 (100 ng/ml) during 24 h. Fas activation did not modify Notch1 expression that remained sequestered in the U-87 MG cells as under basal conditions (Fig. 3D).

Fas activation tends to increase $p75^{NTR}$ intracellular expression. While 20% of $p75^{NTR}$ was detected at the cell surface under a basal condition, activation of the Fas receptor by agonistic anti-Fas mAb 7C11, suppressed $p75^{NTR}$ membranous expression in all U-87 MG cells. Therefore, Fas-activation tended to increase (~10%) the percentage of cells expressing $p75^{NTR}$ in the intracellular compartment, compared to that in basal conditions (a total of 80% of cells expressed intracellular $p75^{NTR}$ vs. 70% under basal conditions) (Fig. 4).

γ-secretase inhibitor modifies Fas-induced apoptosis by $p75^{NTR}$ inhibiting pathway. To demonstrate that $p75^{NTR}$ interacts with Fas-induced apoptosis, the γ-secretase inhibitor was incubated with or without 7C11 (100 ng/ml), and the apoptotic rate was determined by measurement of cytoplasmic soluble nucleosomes (Fig. 5) or membranous staining with Annexin V (data not shown). Fas activation by a 24-h exposure to agonistic anti-Fas mAb significantly induced apoptosis compared to the basal apoptotic level ($p<0.0001$) (Fig. 5). The γ-secretase inhibitor did not modify this apoptotic level in basal conditions. However, concomitant treatment of U-87 MG cells with agonistic anti-Fas mAb and the γ-secretase inhibitor significantly decreased cell apoptosis compared to 7C11 alone ($p=0.007$) (Fig. 5).

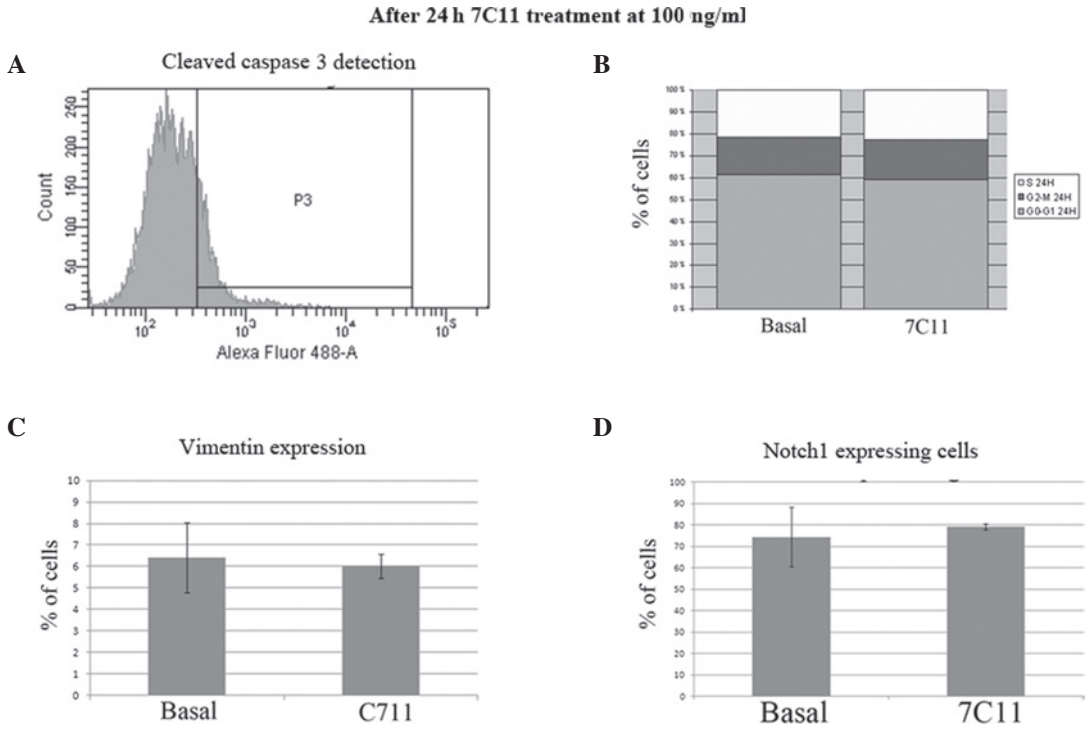

Figure 3. Anti-Fas mAb 7C11 treatment promotes cell apoptosis without modifying the cell cycle, vimentin or Notch1 expression. After a 24-h exposure to anti-Fas mAb 7C11 (100 ng/ml) modification of cell apoptosis, cell cycle, vimentin or Notch1 expression was investigated. (A) Cell apoptosis was evaluated by cleaved caspase-3 expression detected by flow cytometry. Anti-Fas agonistic mAb treatment led to 15% of apoptotic cells in the U-87 MG cell line. (B) The cell cycle was not affected by Fas activation (7C11). Vimentin (C) and Notch1 (D) expression in U-87 MG cells was not modified by Fas activation (7C11).

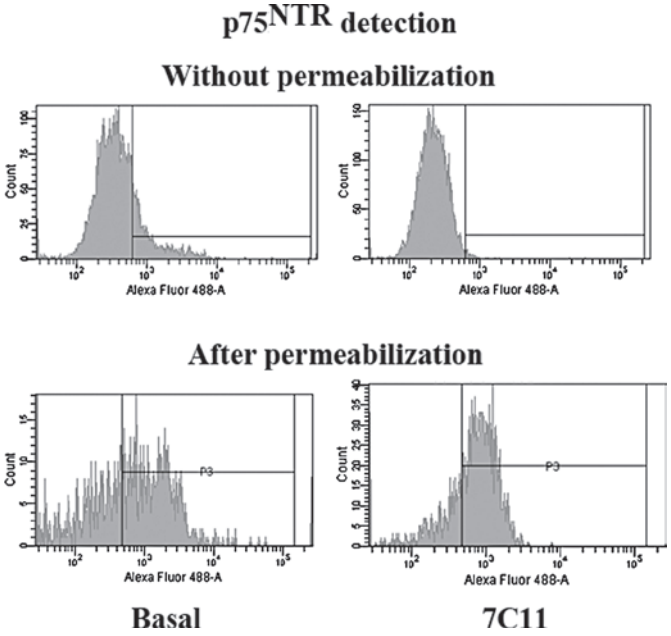

Figure 4. Anti-Fas mAb 7C11 treatment tended to increase p75^{NTR} intracellular expression. Under a basal condition, 20% of U-87 MG cells expressed p75^{NTR} at the membrane level and 70% of these cells expressed p75^{NTR} in the intracellular compartment (left panel). By contrast, Fas activation suppressed p75^{NTR} membrane expression and 80% of cells had an intracellular expression of this receptor (right panel).

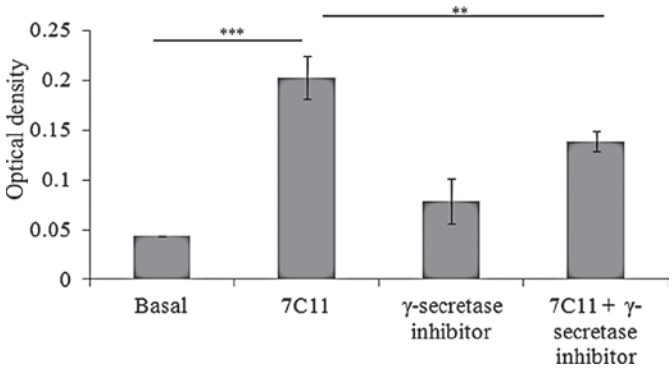

Figure 5. γ-secretase inhibitor decreased Fas-induced apoptosis. U-87 MG cells were treated for 24 h with anti-Fas mAb 7C11, γ-secretase inhibitor or both molecules. The apoptotic rate was determined by measurement of cytoplasmic soluble nucleosomes. Anti-Fas mAb 7C11 promoted cell apoptosis compared to basal condition ($p<0.0001$); by contrast, the γ-secretase inhibitor alone did not significantly modify cell death, whereas it significantly decreased 7C11-induced apoptosis ($p=0.007$). $^{**}p<0.01$; $^{***}p<0.001$.

Discussion

We demonstrated herein that γ-secretase inhibitors decreased Fas-induced apoptosis in the human U-87 MG glioblastoma cell line. These cells expressed the full-length receptor of the Fas death receptor and its activation by an agonistic anti-Fas mAb induced significant apoptosis. Another death receptor, p75^{NTR}, the low affinity receptor for neurotrophins, is known to be linked to the Fas receptor through a common signaling pathway as demonstrated in a neuroblastoma cell line. Indeed, when stimulated alone, i.e., Fas by FasL and p75^{NTR} by the neurotrophin BDNF, each receptor induced cell death, whereas their concomitant activation was found to suppress apoptotic cell death, depending on simultaneous caspase-8 and sphingomyelinase activation (4). However, BDNF-induced cell death through p75^{NTR} activation alone was found to be

inefficient in the U-87 MG glioblastoma cell line containing $p75^{NTR}$ receptor sequestered in Golgi apparatus (13).

By contrast, $p75^{NTR}$ was found to be involved as an activator of tumor invasion, mediated through intra-membranous proteolysis by α and γ-secretase, releasing the intracellular domain that binds the nucleus and activates signal transduction (8,6). This tumor invasion was inhibited by γ-secretase inhibitor *in vitro* and in experimental models in a BDNF-dependent mechanism (7). Nevertheless, this antitumor effect was not evaluated under cell death activation through Fas activation in glioblastoma cells. Indeed, the interaction of Fas and $p75^{NTR}$ (4) during cell-induced apoptosis may be modified. On the other hand, γ-secretase is known to cleave other proteins, including Notch receptors (10).

Notch signaling is well known to play a key role during normal and neoplastic development (15,16). Perturbation of the Notch signaling pathway may often lead to the process of carcinogenesis, including glioblastoma (17). The Notch signaling pathway requires γ-secretase cleavage resulting in Notch intracellular domain translocation in the nucleus and activation of transcription. For this reason, we investigated Notch1 and 2 expression in U-87 MG cells. Indeed, intracellular expression of Notch1, 2 and their ligands was detected in the U-87 MG cell line without membranous location and was not modified by Fas-activation.

In a human neuroblastoma cell line, $p75^{NTR}$ was demonstrated to induce cell death after BDNF stimulation and to interact with the Fas receptor (4). Likewise, our results suggest a potential interaction between Fas and $p75^{NTR}$ in the U-87 MG human glioblastoma cell line. Indeed, γ-secretase inhibitor significantly decreased apoptosis induced through Fas activation. These results suggest that γ-secretase is implicated in the cell death induced by Fas activation through $p75^{NTR}$ cleavage and signaling. Such $p75^{NTR}$ involvement in apoptosis was described in primary neuronal cell cultures in which $p75^{NTR}$ cleavage by γ-secretase resulted in nuclear translocation of the intracellular domain and apoptotic cell death (18).

The present results are in agreement with Fas and $p75^{NTR}$ interactions in glioblastoma cells. Therefore we hypothesized that the γ-secretase inhibitor diminishes Fas-induced apoptosis depending on its interaction with $p75^{NTR}$ through its intracellular domain. Such mechanisms interfering with Fas activation will be of great importance in the search for cell death-inducing treatment. Indeed, whereas glioblastoma cells are spontaneously resistant to cell death induction, it was previously demonstrated that a combination of Fas activation with concomitant etoposide plus dexamethasone exhibits antiproliferative and antitumor properties in experimental glioblastoma in the nude rat (3). We hypothesize that Fas and $p75^{NTR}$ activation are of major importance in apoptosis induction of glioma cells, and that the γ-secretase inhibitor may interfere with Fas-activating treatment.

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