Cancer stem cells from human glioma cell line are resistant to Fas-induced apoptosis

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Abstract. Glioblastoma is the most common primary brain tumor, characterized by its resistance to treatments. To define efficient therapy, the origin of tumor-forming cells needs to be elucidated in order to search for new therapeutic pathways. The objective of this study was to determine the different cell populations constituting a human glioblastoma cell line, U-87 MG and their sensitivity to apoptosis induced through the activation of Fas, a membranous death receptor. By a cell sorting method, the sedimentation field flow fractionation, two major cell subpopulations were identified, a most differentiated cell fraction, containing large and adherent cells, sensitive to Fas-induced apoptosis and another one, characterized by small cells forming aggregates, expressing CD133, a marker of stem cells and more resistant to Fas-activated apoptosis. By using a selective method of culture, adapted for neural stem cell cultures, we have verified that the U-87 MG cell line contained cancer stem cells similar to the immature ones obtained by the cell sorting method. Interestingly, while these tumor stem cells, expressing CD133, were resistant to Fas-induced apoptosis, monomeric form of Fas protein was detected predominantly in these cells. In contrast, the most mature cells, responsive to Fas-activated apoptosis, contained oligomeric aggregates of Fas protein, a pre-signalling form of the Fas receptor, essential for the initiation of apoptosis through its activation. These results suggest that these immature stem cells in glioma could be an important factor of resistance to chemotherapy requiring apoptosis through Fas signalling system. Indeed, future strategies of treatment, inducing differentiation of these stem cells need to be considered to enhance therapeutic efficiency.

Introduction

Glioblastoma multiform is the most common malignant primary brain tumor in adults and it is among the most lethal of all cancers (1). Gliomas are particularly invasive tumors and their prognosis remain very poor despite surgery-, radio- and chemotherapy (2), with a mean survival <1 year. Glioma cells are characterized by their resistance to induce apoptosis and by some features of immature cells. The neural stem-cell model for the origins of gliomas shed light on their heterogeneity (3). Recently, self-renewing, multipotent cells expressing the CD133 surface cell-marker were isolated from human gliomas and transplanted into adult mouse brain, where they recapitulated the parents tumor's histology (4). These findings suggest that gliomas are both initiated and maintained by a small fraction of cancerous neural stem-cells. Therefore, CD133+ cells are obviously considered as a potentially relevant target tumor cell subpopulation for therapy in the context of the whole tumor mass (4).

Apoptosis plays an important role in regulation of cellular activities in eukaryotes (5), and its deregulation contributes to the pathogenesis of several diseases (6). The Fas receptor (APO-1, CD95) is a member of the tumor necrosis factor (TNF)/nerve growth factor death receptor superfamily and its monomeric form is a 45-52-kDa type I transmembrane protein (7). Fas extracellular domain contains three cystein-rich repeats and two of them bind the Fas natural ligand, FasL (8). Their disruption leads to the loss of ligand binding and effector's signalling (9). It was first demonstrated that following engagement with FasL or agonistic anti-Fas monoclonal antibody (mAb), Fas monomers trimerize, which leads to the recruitment of the intracellular death domain and to a downstream activation of the death-inducing signalling complex (10). Subsequently, the adaptor protein Fas-associated death domain interacts with Fas cytoplasmic death domain and recruits procaspase-8, which can thereby self-activate. Cleavage of downstream substrates by caspase-8 rapidly triggers cell death (11). More recently, Fas trimerization was shown to occur also without FasL binding. This preassembly of Fas monomers, named the pre-ligand assembly domain is required for the subsequent clustering of Fas receptor after FasL ligation (12). Fas polymerization leads to sodium dodecyl sulfate (SDS) - stable high molecular weight aggregates that are easily detected by SDS-polyacrylamide gel electrophoresis.
Cells were grown in 25 cm² flasks (Nunc Fisher Bioblock) supplemented with 10% decomplemented foetal calf serum (FCS), 1.5 g/l L-glutamine (2 mM) and fungizone (0.1%) (Gibco BRL, Life Technologies, Paisley, UK) in MEM with Earle’s salts (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% decomplemented foetal calf serum (FCS) and 1.5 g/l L-glutamine (2 mM) and fungizone (0.1%) (Gibco BRL). Cells were maintained in MEM with Earle’s salts (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% decomplemented foetal calf serum (FCS) and 1.5 g/l L-glutamine (2 mM) and fungizone (0.1%) (Gibco BRL). Cells were grown in 25 cm² flasks (Nunc Fisher Bioblock Scientific, Illkirch, France) at 37°C in a humidified 5% CO₂-95% air incubator.

Freshly versenized, 10⁶ cells were seeded onto flasks (25 cm²) for 3 days before SdFFF analysis. Cell viability was determined by Propidium Iodide staining: cell concentration was adjusted to 2.5x10⁶ cells/ml in phosphate buffered saline (PBS) before SdFFF analysis. After SdFFF sorting, control and eluted cells were seeded (10⁵ cells/well) in 24-well-plates (Nunc, Fisher Bioblock Scientific). For light microscopy, cultured cells were examined under phase-contrast microscopy (magnification x400). Photos were taken with a Nikon camera (Champigny/ Marne, France).

To obtain neurosphere-like cells, U-87 MG cells were cultured in serum-free medium (23) containing DMEM/F12, 30% glucose (Sigma Aldrich, Saint-Quentin-Fallavier, France), 1 M HEPES buffer (Sigma), progesterone (Sigma), putrescine (Sigma), B27 growth supplement (Invitrogen), 20 ng/ml EGF (Sigma), 20 ng/ml FGF (Sigma), Insulin-Transferrin-Sodium Selenite Supplement (Roche Diagnostics, Meylan, France) and heparin (Sigma). After a 4-day culture, U-87 MG formed primary neurospheres. Primary neurospheres were dissociated and cells were seeded to form secondary neurospheres, cultured during 48 h before immunostaining and apoptosis studies.

**Immunocytofluorescence staining.** For immunofluorescence studies, cells were grown on glass coverslips in a 24-well plate. After two washes in PBS, the cells were fixed for 15 min in 4% paraformaldehyde in PBS, washed twice in PBS and permeabilized 30 min with a solution of Triton X-100 0.1%-PBS at room temperature. Then, the cells were washed twice in PBS and incubated in PBS-4% bovine serum albumin (BSA) for 2 h at room temperature. Cells were incubated overnight at 4°C with primary antibodies (Abs) diluted in PBS-BSA. The following antibodies were used: a mouse anti-vimentin monoclonal Ab (mAb) (clone RV202, 1:200 dilution, Santa Cruz Biotechnology, Heidelberg, Germany), a rabbit polyclonal anti-CD133 (clone RB 1784, 1:100 dilution, Abgent, San Diego, CA), or anti-glial fibrillary protein (GFAP) (1:500 dilution, DakoCytomation, Trappes, France). Negative controls were done with cells incubated with irrelevant normal rabbit or mouse IgG (Santa Cruz).

Cells were washed twice in PBS and incubated with 1 μg/ml Alexafluor 488- or 596- conjugated anti-mouse or anti-rabbit immunoglobulin (Ig) Abs (Invitrogen) for 120 min at 4°C. After two further washes in PBS, cells were mounted in glycerol-gelatin medium (Sigma-Aldrich) and fluorescence was analyzed using a Leica DMRX microscope (Leica Microsystems, Rueil-Malmaison, France).

**Flow cytometry analysis.** Cells were harvested, washed with PBS, fixed and permeabilized with Intrastain (DakoCytomation), before staining with the Abs recognizing vimentin, CD133 or GFAP purchased as above. Fas expression was analyzed with a rabbit anti-Fas polyclonal Ab (clone C-20, Santa Cruz) specific of the intracellular part of the receptor, at a dilution of 1:100 in PBS for 30 min at 4°C, and revealed by Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Ig (Santa Cruz) at a dilution of 1:200 in PBS for 30 min at 4°C before analysis by flow cytometry (Beckman FC 500, Beckman Coulter Villepinte, France). Moreover

**Materials and methods**

**Cell line and cell culture.** The U-87 MG human glioblastoma cell line was obtained from American Type Culture Collection (ATCC, LGC Promochem SARL, Molsheim, France). The cells were maintained in MEM with Earle’s salts (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% decomplemented foetal calf serum (FCS), 1.5 g/l sodium bicarbonate, 1% non-essential amino acids, sodium pyruvate (2 mM), penicillin (50 U/ml), streptomycin (50 U/ml), L-glutamine (2 mM) and fungizone (0.1%) (Gibco BRL). Cells were grown in 25 cm² flasks (Nunc Fisher Bioblock Scientific, Illkirch, France) at 37°C in a humidified 5% CO₂-95% air incubator.
Apopotosis quantification. After a 48-h culture, cells were treated with 40 ng/ml of anti-Fas agonistic mAb (Fas clone 7C11 sodium azide-free, Beckman Coulter) (24). After a 24-h treatment, apoptosis was analyzed by two methods. One method, measuring cytoplasmic soluble mono- and oligo-nucleosomes released during apoptosis, was based on enzyme-linked immunosorbent assay (ELISA) using antibodies directed against DNA and histones (Cell Death Detection ELISA®PLUS, Roche Diagnostics, Meylan, France) according to the manufacturer's protocol to quantify apoptotic index as previously described (25).

Another method used Annexin-V-FITC staining (Beckman Coulter) which indicates phosphatidylserin translocation to the cell surface in apoptotic cells. This staining was associated to those of propidium iodide (PI, red fluorescence), a DNA-intercalating agent that reflected the membranous permeability as observed during necrosis or at the latter stage of apoptosis. Cells were analyzed by flow cytometry (Beckman FC 500).

SdFFF device and cell elution conditions. SdFFF separation device used in this study derived from those previously described and schematized (26,27). The apparatus consists of two 938x40x2 mm polystyrene plates, separated by a mylar® spacer in which the channel was curved. Channel dimensions were 818x12x0.175 mm with two 50 mm V-shaped ends. The measured total void volume (channel volume + connecting tubing + injection and detection device) was 1792±2.00 μl. Void volume was calculated after injection and elution time determination of a non-retained compound (0.10 g/l of benzoic acid, UV detection at 254 nm, n=6). The channel rotor axis distance was measured at r=14.82 cm. A heterogeneous model which contains two cell-types: adherent and elution time determination of a non-retained compound.

Western blotting. After SdFFF analysis cells were lysed in RIPA lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, and 20 μg/ml aprotinin) containing protease inhibitors (Complete Mini; Roche Diagnostics).

Equal amounts of proteins from cell lysates (40 μg/lane) were separated on NuPAGE 4-12% SDS-polyacrylamide gels (Invitrogen) under denaturing conditions and transferred onto nitrocellulose sheets (Hybond, GE Heathcare, Orsay, France). Non-specific binding sites were blocked for 2 h with 5% non-fat dry milk in TBS containing 0.1% Tween-20. After overnight incubation at 4°C with anti-Fas rabbit polyclonal Ab (clone C20, Santa Cruz,) and anti-Fas mAb (clone B10, Santa Cruz) (1/200 dilution) (both directed against intracellular part of the receptor), membranes were incubated with horseradish peroxidase (HRP)-conjugated Abs to mouse or rabbit Ig (1/2000 dilution, DakoCytomation) for 60 min at room temperature and revealed by chemiluminescence (ECL reagent, Amersham Life Science, Inc., Buckinghamshire, UK). Protein loading control was performed with rabbit anti-GAPDH Ab (Santa Cruz Biotech). Western blots were scanned using a bio-imaging system (Genesnap, Syngene, Cambridge, UK).

Statistical analysis. Mean and standard errors (SEM) were evaluated in three independent experiments. Statistical analysis was carried out by ANOVA test using Statview (Ver. 5.0). A p<0.05 (Fisher's PLSD test) was considered as significance.

Results

Characterization of U-87 MG cells. U-87 MG cell line is a heterogeneous model which contains two cell-types: adherent cells and small sphere cells forming aggregates (Fig. 1A). To characterize the U-87 MG cell line we used Abs recognizing three different markers: GFAP (an astrocytic cell marker), vimentin (a weakly differentiated cells marker) (29) and CD133 (a stem cell marker) (30).

The majority of U-87 MG cells were stained with anti-GFAP Ab (Fig. 1B); this result was confirmed by flow cytometry which indicates phosphatidylserin translocation to the cell surface in apoptotic cells. This staining was associated to those of propidium iodide (PI, red fluorescence), a DNA-intercalating agent that reflected the membranous permeability as observed during necrosis or at the latter stage of apoptosis. Cells were analyzed by flow cytometry (Beckman FC 500).
cytometry (Fig. 1F). The anti-vimentin staining demonstrated that all cells were positive (Fig. 1C and G). Moreover, the strongest intensity of vimentin staining seemed to be in small sphere cells (Fig. 1C) and in cells present in aggregates (Fig. 1D). Finally, by using CD133 marker, we detected some CD133+ cells in aggregates (Fig. 1E), whereas these cells were not detected by flow cytometry in total cell population (Fig. 1H).

Fas-induced apoptosis in total U-87 MG cells. To define the potential apoptotic functions of Fas receptor in glioblastoma, apoptosis induced through the Fas pathway was evaluated in U-87 MG cells, expressing Fas receptor as assessed by flow cytometry analyses, by using antibodies directed either against intracellular or extra-cellular part of Fas receptor (Fig. 2A and B).

After a 48-h culture, Fas activation was performed by a 24-h incubation of cells with an agonistic anti-Fas mAb (7C11 - 40 ng/ml). Apoptosis was significantly induced through Fas activation as detected by two methods. Indeed, the measurement of soluble nucleosomes by ELISA showed an increase of apoptotic ratio of cells incubated with anti-Fas mAb (Fig. 2C) in comparison to cells cultured in basal conditions (p=0.0059). These results were confirmed by flow cytometric analysis showing the percentage of apoptotic cells, only labelled by annexin-V (Fig. 2D represents one of the three independent experiments). However, this method showed that only a small percentage (18.43%) of cells of the total population underwent to apoptosis in presence of agonistic anti-Fas mAb, since 9.21% where stained in basal conditions (p=0.008). Interestingly, this second method was able to distinct a cell subpopulation sensitive to Fas-induced
apoptosis, among the prominent other one resistant to Fas-activated apoptosis. Therefore, the following results focused:
i) on the isolation of these two cell populations and ii) on their apoptotic response through Fas activation pathway.

Distinct cell populations identified by SdFFF elution. To isolate and study these two subpopulations, we used an innovative tool called Sedimentation field flow fractionation (SdFFF). Fig. 3 displayed a representative fractogram of U-87 MG cells. The first part of fractogram corresponded to the void volume peak (R_{obs} ≈ 1, retention ratio R_{obs} = void time versus retention time = t_0/t_R) (31) while the second and broader part, corresponded to the cell elution profile.

Elution conditions have been experimentally determined to allow separation of larger cells from void volume peak, reducing selective cells subpopulation lost. Then, a R_{obs} value was specifically measured for the more retained cells corresponding to the last fraction and smaller cells (Fig. 3). R_{obs} F3 = 0.351±0.021 (n=6). As previously described (32,33), the absolute R_{obs} value particularly depends on culture conditions, thus for U-87 MG cells the number of cell passages which should range from 2 to 8. At constant flow rate (0.80 ml/min), according to the 'Hyperlayer' elution mode description (31,33-39), the R_{obs} value for F3 is field dependant: R_{obs} = 0.36±0.012 (n=3) at 20.00 g. By using the following equation (40):

$$R = \frac{6s}{\omega}$$  (1)

in which R is the retention ratio, ω is the channel thickness (175 μm), and s the distance from the center of the focused zone to the channel wall (40), we calculated the approximate average cell elevation (s) using R_{obs} values: s = 10.24 μm. The mean cell diameter for F3 was 12.74±0.10 μm (n=3, Coulter Counter®). Thus, radii of last eluted cells were less than the approximate average cell elevation value: r = 6.37 < s =10.24 μm. Finally, after total cell elution, when the external field was turned off (mean external field = 0.00 g, end of rotating, ER) (Fig. 3), we observed a residual signal (RP, Fig. 3) corresponding to the reversible particle release from the accumulation wall. Four cell fractions were collected: i) the total peak fraction (TP) which corresponded to the total U-87 MG eluted population; and ii) peak fractions 1, 2 and 3 (PFN) which were time-dependent collected fractions of retained cells (Fig. 3).

Morphological observations. After SdFFF elution cells were cultured for 4 h and observed by optical microscopy. Fig. 4A show that TP contained two cell types: adherent cells and small sphere cells. PF1 was composed by a majority of adherent cells (Fig. 4B), PF2 was a mix between the two cells type (Fig. 4C) whereas small sphere cells were observed in PF3 (Fig. 4D). These different observations demonstrated that SdFFF could separate two cell types: large adherent cells in PF1 and small sphere cells in PF3. Moreover, size and density of the four fractions studied by flow cytometry (Fig. 5)
showed that PF3 was enriched with small cells, confirming optical microscopic observations.

**Characterization of SdFFF fractions by flow cytometry.** Three markers, N-CAM, vimentin and CD133 have been used for characterization. First, N-CAM was used to characterize the adherent cells in glioma. Global expression of N-CAM, observed in the TP revealed that 56.6% of cells were N-CAM+ (Fig. 6A). Furthermore, PF1 and PF2, the two enriched-adherent cell fractions, contained 74 and 78.6% N-CAM+ cells, respectively (Fig. 6B and C), whereas in PF3, 59.9% N-CAM+ cells were detected (Fig. 6D).

Then, vimentin, expressed in cells in proliferation and differentiation and generally characteristic of the weakly differentiated cells (29) was assessed. The total expression of vimentin, analyzed in the TP (Fig. 7A) was similar to those in PF1 and PF2 cells (Fig. 7B and C) while vimentin expression was more strongly expressed in PF3 (Fig. 7D). These results demonstrated different vimentin expression levels between the three fractions with highest level in PF3, containing the most immature cells as hypothesized by morphological features.

Indeed, we analyzed this cell fraction with anti-CD133 Ab, CD133 being described as a stem cell marker (30). CD133 staining was reported to be associated to brain stem tumor-initiating cells, that reconstitute the original tumor in vivo and after transplantation (4). By flow cytometry, CD133 expression was detected in a small percentage (11.8%) of total cell line and of the TP (Fig. 8A). Interestingly, PF3 was strongly enriched with 72% CD133+ expressing cells (Fig. 8D), in contrast to other fractions, PF1 and PF2, containing 12.95 and 25% CD133+ cells, respectively (Fig. 8B and C). Together, these markers revealed that the F3 cell subpopulation contained cells expressing immature (vimentin+) and stem cell markers (CD133+), in contrast to F1 cell sub-population, containing most mature (N-CAM+) cells.

**U-87 MG cells cultured in serum-free medium.** To obtain CD133+-enriched cells, U-87 MG cells were cultured in FCS-free medium (23). Contrasting with the two different cell types detected in U-87 MG cell line cultured with FCS-containing medium (adherent cells and cells forming aggregates) (Fig. 9A), U-87 MG cells cultured in serum-free medium displayed neurosphere-like aggregates (Fig. 9B and C). Study of cell size determined by flow cytometry showed that cells in FCS-free medium (Fig. 9E) were smaller than cells cultured in normal (FCS-containing) medium (Fig. 9D). In these conditions, the staining of cells with anti-CD133 Ab demonstrated that 34.38% cells were CD133+ (Fig. 9F and G) a percentage significantly higher than in FCS-containing medium (p=0.0074). Therefore, U-87 MG cells cultured in FCS-free medium formed neurospheres containing CD133+ stem cells.
Oligomeric aggregate Fas receptor detected in the eluted fraction PF1 (most mature cells) but not in PF3 (immature cells). Fas receptor was expressed in the different cell sorting fractions as shown by Western blotting (Fig. 10A). However, some differences of Fas protein oligomerization were identified among the eluted cell fractions. Whereas Fas monomer (45 kDa) was detected in the 3 fractions and in the whole cell population (TP), a higher molecular weight protein of 120 kDa was the predominant form detected in the whole cell and PF1 protein lysates. In contrast, PF3 proteins, containing mostly the monomeric form. This high molecular weight of Fas receptor was detected with an antibody directed against the intracellular part of the receptor, available to detect the aggregated Fas receptor (14), described as the effective form of pre-associated Fas receptor, able to induce apoptosis (10).

Different sensitivity to Fas-induced apoptosis depending on cell maturation. The apoptotic response to a 24-h incubation with agonistic anti-Fas mAb (7C11-40 ng/ml) was different between
the eluted cell fractions as assessed by annexin-V staining (Fig. 10B). Indeed, a significant difference was detected between the 3 SdFFF fractions: most mature cells (PF1 sub-population) exhibited the highest apoptotic rate (38%), whereas, sensitivity to apoptosis of immature cells (PF3 sub-population), was significantly lower (25%) (p=0.0314, Fig. 10B). An intermediate stage of sensitivity to apoptosis (30%) was detected in eluted cells from intermediate fraction, PF2, which was similar to those obtained in whole cells, obtained after cell sorting (TP, 32%). In contrast, basal apoptosis (without Fas activation) of total cells (contained in PT) after cell sorting by SdFFF was lower, ~20% (data not shown).

Likewise, stem cells forming neurosphere-like, obtained in serum-free medium, were insensitive to a 24-h incubation with the agonistic anti-Fas Ab (7C11 - 40 ng/ml) (17.33±2.14% of apoptotic cells). Apoptotic ratios were similar to those obtained in cells cultured in basal conditions (15.36±1.42%, p>0.05) (Fig. 10C). These results demonstrated that cells present in neurospheres were resistant to Fas-induced apoptosis which assessed results obtained in SdFFF eluted cells.

**Discussion**

The aim of this study was to determine the sensitivity difference of human glioma cells to Fas-induced apoptosis...
among the maturation stage of cells contained in the human U-87 MG line. Such objective was supported by the hypothesis that resistance of gliomas to treatment could be due to some difference in sensitivity to apoptosis induction among cells contained in the U-87 MG glioma cell line. The induction of apoptosis through the death receptor, Fas, by an agonistic anti-Fas mAb (7C11) studied by Western blotting of U-87 MG cell proteins with anti-Fas mAb directed against intracellular part of Fas receptor (A), detection of monomeric form of Fas (45 kDa) in the three eluted cell fractions (F1, F2, F3) as in the whole population. The oligomeric aggregates of Fas receptor (120 kDa) were not detected in F3. GAPDH detected by specific Ab, is the control of protein loading. Apoptosis induced by agonistic anti-Fas mAb (7C11) studied by annexin-V staining revealed that apoptosis was significantly (*) higher in F1 cells than in F3 (B). Cells cultured in serum-free medium were resistant to 7C11-induced apoptosis (C) as assessed by similar apoptotic percentage in cells maintained in serum-free medium without Fas activation (control).

As previously described (22,27,33,39,44,45), cell species are eluted under the Hyperlayer model, a size/density dependent cell elution mode (31,33-39), which predicts that large and weaker cells are focused in faster streamlines to be eluted first. Hyperlayer elution mode allowed, by limiting cell-accumulation wall interactions, a cell elution which: i) respected repeatability, reproducibility and recovery; ii) respected cell functional integrity; iii) provided high levels of short and long-term viability (no apoptosis induction); iv) respected the maturation and differentiation stages; and finally; v) enhanced better sub-population sorting and characterization (20,22,26,27).

U-87 MG cell line appeared as a heterogeneous population (Fig. 4), leading, as for neuroblastoma cell line (26), to complex fractograms (Fig. 3) in contrast to many other cell lines (22,27,42,43). According to results concerning: i) the particle elution away from the accumulation wall (s>r); ii) field dependence of the Robs value; and iii) the limited interactions between cells and accumulation wall leading to low reversible cellular release (RP, Fig. 3); we can suppose that U-87 MG cells were eluted under Hyperlayer elution mode as it has been demonstrated for other cell models (22,26,27).

However, from a biological point of view, we did not know if cell sorting of the different U-87 MG sub-populations of interest was achieved. Four cell fractions were collected: the total peak fraction (TP) which corresponded to the total U-87 MG population eluted, used as control of whole cells; and three peak fractions 1, 2 and 3 (PFn) which are the time-dependent collected fractions of the retained peak profile. Four cell fractions were collected: the total peak fraction (TP) which corresponded to the total U-87 MG population eluted, used as control of whole cells; and three peak fractions 1, 2 and 3 (PFn) which are the time-dependent collected fractions of the retained peak profile. Cell culture, morphological observation, specific antigen expression and apoptosis analysis were performed in each fraction in order to characterize these different sub-populations and to determine their respective apoptosis sensitivities to Fas activation.

Figure 10. Comparative effect of Fas activation depending on maturation stage of cells in SdFFF isolated fractions and in glioma neural stem cells. Western blotting of U-87 MG cell proteins with anti-Fas mAb directed against intracellular part of Fas receptor (A), detection of monomeric form of Fas (45 kDa) in the three eluted cell fractions (F1, F2, F3) as in the whole population. The oligomeric aggregates of Fas receptor (120 kDa) were not detected in F3. GAPDH detected by specific Ab, is the control of protein loading. Apoptosis induced by agonistic anti-Fas mAb (7C11) studied by annexin-V staining revealed that apoptosis was significantly (*) higher in F1 cells than in F3 (B). Cells cultured in serum-free medium were resistant to 7C11-induced apoptosis (C) as assessed by similar apoptotic percentage in cells maintained in serum-free medium without Fas activation (control).
volume. Nevertheless, the study of Fas-activating apoptosis in SdFFF eluted fractions, showed a significant difference between PF1 and PF3. Indeed, cells sorted in PF1 (large adherent cells, and CD133) seem to be the most sensitive to the apoptosis induced by Fas receptor activation while cells in PF3 (small sphere cells, most undifferentiated and CD133+) are the most resistant to Fas-induced apoptosis. A previous study demonstrated that CD133+ cells were responsible of preservation and proliferation of the tumor in glioma (3). Therefore, this tumor cell sub-population could be implicated in the resistance of glioblastoma to Fas-induced apoptosis. The culture in defined serum-free medium was described to be a selective growth medium of enriching neural stem cells in cultures from normal brain tissues (46) as brain tumor (23). In comparison, SdFFF cell sorting also performed the isolation of cancer stem cells, expressing CD133+. Furthermore, cells obtained by this selective culture method exhibited resistance to apoptosis, similar to those sorted in PF3. Therefore, we could assess that SdFFF is able to quickly separate immature cancer stem cells, expressing CD133+, and adherent, N-CAM-expressing cells. N-CAM expression in gliomas was described to enhance adhesion and reduce cell invasion and tumor growth (47) and is expressed by normal differentiated astrocytes (48). These adherent cells, eluted in PF1, contained oligomeric aggregates of Fas receptor, the initial signalling form of Fas (10) and were sensitive to apoptosis through Fas pathway. In contrast CD133+ PF3 cells contained mostly the monomeric form of Fas receptor and were resistant to apoptosis induced through Fas activation.

As glioblastomas were described to be due to abnormal differentiation of neural stem cells belonging to the sub-ventricular zone (3), it seems of first importance to focus research on new therapeutic pathways to eradicate cancer stem cells, isolated from tumor patients. The conditions for inducing differentiation of these immature cells need to be further defined in order to re-establish the response of gliomas to chemotheraphy.

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References

12. Bertrand et al: FAS RECEPTOR AND GLIOMA STEM CELLS 726

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References

4. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hilde...


