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Original Articles

Optimized dissociation protocol for isolating human glioma stem cells from tumorspheres via fluorescence-activated cell sorting

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ABSTRACT

Fluorescence-activated cell sorting (FACS) based on the surface marker CD133 is the most common method for isolating glioma stem cells (GSCs) from heterogeneous glioma cell populations. Optimization of this method will have profound implications for the future of GSC research. Five commonly used digestion reagents, Liberase-TL, trypsin, TrypLE, Accutase, and non-enzymatic cell dissociation solution (NECDS), were used to dissociate glioma tumorspheres derived from two primary glioma specimens (091214 and 090116) and the cell lines U87 and T98G. The dissociation time, cell viability, retention of CD133, and stemness capacity were assessed. The results showed that single cells derived from the Liberase-TL (200 μg/ml) group exhibited high viability and less damage to the antigen CD133. However, the efficiency of NECDS for dissociating the tumorspheres into single cells was fairly low. Meanwhile, the use of this digestion reagent resulted in obvious cellular and antigenic impairments. Taken together, Liberase-TL (200 μg/ml) is an ideal reagent for isolating GSCs from tumorspheres. In contrast, the use of NECDS for such a protocol should be carefully considered.

Introduction

Malignant glioma is the most frequent central nervous system tumor and is extremely lethal. Increasing evidence demonstrates that GSCs, the rare subpopulation possessing self-renewal, multipotency, and tumorigenicity in the heterogeneous tumor mass [1], are responsible for the initiation, invasion, drug-resistance, and recurrence of malignant glioma [2,3]. Therefore, efficiently and reliably isolating GSCs is the starting point for a better understanding of the biological characteristics of GSCs and for the development of novel therapeutic regimens.

Although there are many strategies for isolating GSCs [4], fluorescence-activated cell sorting (FACS) is the most commonly used method [1,5] to separate living cells based on functional properties or cell-surface antigens with different fluorescent intensity. CD133, a glycoprotein possessing five transmembrane domains, has previously been used for obtaining human hematopoietic and neural stem cells [6–8] and then for isolating GSCs in 2004. These $CD133^+$ glioma cells exhibit tumor stem cell properties *in vitro* and *in vivo* [9,10]. In addition, the culture of tumorspheres in serum-free media is an important method for enriching GSCs [11]. Compared with the conventional adherent culture condition, these tumorspheres contain a higher proportion of $CD133^+$ glioma cells [12,13]. Although FACS has been widely used to separate $CD133^+$ GSCs from glioma tumorspheres, a variety of factors including some experimental procedures are likely to influence the efficacy of this method [14]. In particular, the dissociation of tumorspheres into a single-cell suspension is the first and most critical step of FACS based on CD133 expression. The digestion reagents used in this procedure will profoundly influence the cellular viability and antigen retention of single cells. Thus, optimization of the dissociation protocol will improve the efficiency and reliability of FACS for isolating $CD133^+$ GSCs from tumorspheres.

In this study, five digestion reagents, Liberase-TL [15], trypsin [16], TrypLE [17,18], Accutase [19], and non-enzymatic cell dissociation solution (NECDS) [20], were assessed for their ability to dissociate the tumorspheres, which were derived from 2 primary glioma specimens and the cell lines U87 and T98G, into single-cell suspensions. The dissociation efficiency was highest in the Liberase-TL (200 μg/ml) group, and the single cells derived from this group displayed robust cellular viability and CD133 antigen retention.

Materials and methods

Cell lines and culture

The human glioblastoma cell lines U87 and T98G were purchased from the American Type Culture Collection (ATCC). Primary human glioma cells, 091214 and 090116,

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were obtained from the specimens of two glioma patients, respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone) containing 10% fetal bovine serum (FBS, HyClone), 2 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 1% nonessential amino acids. To obtain tumorspheres, glioma cells were seeded in 6-well ultra-low-attachment plates at 5000 cells/well containing 1 ml serum-free neural stem cell medium, which is DMEM/F12 (HyClone) supplemented with 2% B27 (Invitrogen), 200 ng/ml recombinant human epidermal growth factor (rhEGF) (Sigma-Aldrich), and 20 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich). The medium was changed after 3 days. Tumorspheres were cultured for 8 days for the subsequent tests. All tumor cells were cultured at 37 °C in a 5% CO_2 water-jacketed incubator.

Dissociation of tumorspheres

Tumorspheres were harvested and then washed with Dulbecco's phosphate-buffered saline (D-PBS) twice, then evenly separated into different tubes. The tumorspheres in four tubes were resuspended with Accutase (Millipore), trypsin (Solarbio), TrypLE (Gibco), and NECDS (Applygen). In the remaining tubes, various concentrations (50, 100, 200 and 300 μg/ml) of Liberase-TL (Roche) were added. Incubations were performed at room temperature without additional humidification and placed in a 37 °C rotator oven during digestion. The standard dissociation time was 10 min. However, in some experiments, the dissociation time was varied for each enzyme preparation. Samples were spun at 200 × g for 5 min and then resuspended in fresh D-PBS without enzyme for further use. For dissociation time span tests, samples were dynamically observed using an inverted phase contrast microscope. After tumorspheres were dissociated into individual cells, the dissociation time span was recorded. Each test was repeated for a total of at least three times.

Flow cytometry

For flow cytometric analysis, cells were resuspended in a flow cytometry buffer consisting of D-PBS, 1.55 mg/ml glucose, and 0.1% bovine serum albumin (BSA, Sigma-Aldrich). Cells were counted and diluted to a density of 1×10^5 cells/ml. For viability analysis, the Fluorescein isothiocyanate (FITC) Annexin-V Apoptosis Detection Kit (BestBio) was used. Annexin-V-FITC (5 μl) was added in 400 μl cell suspension and then incubated for 15 min at 4 °C; for the final 5 min of this incubation, propidium iodide (PI) was added to a final concentration of 50 ng/ml. Higher concentrations of either dye substantially shifted the fluorescence of the entire population during FACS analysis. We analyzed the stained cells by BD FACSCalibur. For CD133 analysis, antibodies against human CD133/2 (clone 293C3-APC, Miltenyi Biotec.) were used. Antibodies were titrated over a semi-log scale to determine the appropriate dilution and were incubated on ice for 30 min. Then, cells were washed twice and resuspended in D-PBS. Finally, cells were analyzed on a FACSAria II flow cytometer (BD Biosciences). Background fluorescence was measured using cells labeled with the isotype control (APC Mouse IgG2b, Miltenyi Biotec.), which sets gating parameters between positive and negative cell populations. Cell aggregates and small debris were excluded from analysis on the basis of side scatter (measuring cell granularity) and forward scatter (measuring cell size); dead cells were excluded from analysis on the basis of viability dye fluorescence. Fluorescent intensities for cells in the population were point-plotted on two-axis graphs or histograms using CellQuest and FACSDiva softwares (BD Biosciences) and the FlowJo software (Tree Star).

Western blotting

U87 tumorsphere cells were dissociated in Liberase-TL (200 μg/ml), Accutase, trypsin, TrypLE, or NECDS for 30 min and then centrifuged at 300 × g for 3 min to obtain cells and digested supernatant. The proteins in the digested supernatant were harvested using Amicon Ultracel-30K Centrifugal Filter Units (Millipore). Cell lysates were prepared by incubation with lysis buffer (40 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.1% Nonidet P-40) supplemented with protease inhibitors. Then, proteins in cell lysates or digested supernatant were separated using SDS-polyacrylamide gels (Beyotime Technologies) and transferred to PVDF membranes. Each membrane was blocked with 5% nonfat dry milk in Tris-buffered saline and incubated with primary antibodies overnight at 4 °C. Blots were developed with a peroxidase-conjugated secondary antibody, and proteins were visualized using enhanced chemiluminescence (ECL) procedures (Thermo), according to the manufacturer's protocol. The primary antibodies used included anti-CD133 (1:200, Miltenyi Biotec) and anti-GAPDH (1:300, Bioworld).

Colony and sphere formation

Five methods were used to thoroughly dissociate tumorspheres to prepare single-cell suspensions. For the colony formation assay, dissociated cells were plated at 400 per well in DMEM supplemented with 10% FBS in 24-well plates. After incubation for 10 days at 37 °C with 5% CO_2, the number of colonies was counted manually. For analysis of sphere formation, dissociated cells were seeded into serum-free neural stem cell media at a density of 100 per well in 96-well plates. Then, after 14 days, the tumorspheres were counted using an inverted phase contrast microscope. The seeding of cells onto the plates was manipulated by flow cytometry.

Immunostaining

Cells were plated on glass coverslips and then fixed with 4% paraformaldehyde and incubated with antibodies against glial fibrillary acidic protein (GFAP, rabbit polyclonal, Abcam), β-tubulin III (rabbit polyclonal, Abcam), or myelin basic protein (MBP, mouse monoclonal, Abcam). Appropriate secondary antibodies (Cy3-red goat anti-rabbit or Alexa Fluor 488 goat anti-mouse, Invitrogen) were used. The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) to reveal nuclei. The staining was detected by laser-scanning confocal microscopy (SP-5, Leica).

Table 1
Dissociation efficiency and cell viability assays.

Cell	Dissociation	Time (s)	Intact cells (%)	Main PI- (%)	Total PI- (%)	Main Annex V- (%)	Total Annex V- (%)
U87	Lib 50	101.70 ± 12.58	59.60	88.83	52.95	84.15	50.16
	Lib 100	99.30 ± 10.07	50.30	84.14	42.35	84.12	42.34
	Lib 200	72.30 ± 2.52	64.00	89.70	57.42	88.55	56.68
	Lib 300	70.00 ± 5.00	59.70	88.47	52.83	87.51	52.26
	Trypsin	66.70 ± 5.78	39.90	81.02	32.36	81.56	32.58
	TrypLE	48.30 ± 17.56	53.80	86.34	46.41	87.40	46.98
	Accutase	83.30 ± 15.26	36.40	73.03	26.60	74.70	27.21
	NECDS	656.70 ± 60.28*	34.40	43.27	14.90	48.35	16.65
091214	Lib 50	623.33 ± 28.67	66.04	93.55	61.78	87.5	57.79
	Lib 100	618.30 ± 60.60	66.65	94.01	62.66	84.85	56.55
	Lib 200	470.00 ± 29.44	73.47	95.4	70.09	85.16	62.57
	Lib 300	446.67 ± 28.67	64.59	88.56	57.20	51.19	33.32
	Trypsin	496.67 ± 36.82	70.13	79.04	55.43	47.25	33.14
	TrypLE	423.33 ± 49.22	72.12	79.06	57.02	55.76	40.21
	Accutase	563.33 ± 26.25	64.93	91.79	59.60	85.79	55.70
	NECDS	1528.70 ± 269.45*	52.78	48.24	25.46	20.43	10.78
090116	Lib 50	327.33 ± 44.58	59.08	93.55	55.27	87.5	51.70
	Lib 100	258.67 ± 34.31	60.74	91.2	55.39	71.59	43.48
	Lib 200	215.67 ± 27.64	65.03	93.4	60.74	77.27	50.25
	Lib 300	185.00 ± 26.77	63.46	84.57	53.67	48.92	31.04
	Trypsin	206.33 ± 17.33	62.35	87.95	54.84	76.76	47.86
	TrypLE	216.67 ± 28.67	64.51	78.48	51.01	55.64	36.17
	Accutase	263.67 ± 38.13	60.94	83.81	51.07	50.79	30.95
	NECDS	1074.33 ± 114.53*	46.02	58.71	27.02	40.86	18.80

Numbers indicate percentages of cells. Cytometric gating is as follows: Main, percentage of intact cells as measured by forward/side scatter characteristics (main population); Total, percentage of all detected events, including pyknotic cells, debris and statistical noise (total population).
Abbreviations: PI, Propidium Iodide; Lib, Liberase-TL.
Data are shown as means ± SD; a two-sided Student's t test was used to generate *P* values.
* $P < 0.05$.