Published in final edited form as:

J Neurosci Methods. 2011 May 15; 198(1): 99–102. doi:10.1016/j.jneumeth.2011.03.003.

A novel fixative for immunofluorescence staining of CD133-positive glioblastoma stem cells

Jonathan H. Sherman^{a,*}, Gerard T. Redpath^b, Jan A. Redick^c, Benjamin W. Purow^d, Edward R. Laws^e, John A. Jane Jr.^a, Mark E. Shaffrey^a, and Isa M. Hussaini^b

^aDepartment of Neurological Surgery, University of Virginia, Charlottesville, Virginia, USA

^bDepartment of Pathology, University of Virginia, Charlottesville, Virginia, USA

^cSchool of Medicine, Core Facility, University of Virginia, Charlottesville, Virginia, USA

^dDepartment of Neurology, University of Virginia, Charlottesville, Virginia, USA

^eDepartment of Neurological Surgery, Brigham & Women's Hospital, Boston, Massachusetts, USA

Abstract

Isolation of glioblastoma stem cells requires incubation of tumor cells in a neural stem cell media. Neurospheres containing these glioblastoma stem cells are formed after approximately a five-day period. These cells can then be analyzed for the presence of stem cell markers. Immunofluorescence staining for these markers can serve as a valuable tool for analyzing the intact neurosphere directly in stem cell media. Here we present the use of a novel fixative (1,4-benzoquinone) for immunofluorescence staining of neurospheres.

Keywords

1,4-Benzoquinone; Immunofluorescence; CD133; Glioblastoma stem cells

1. Introduction

The cell surface marker CD133 has been identified and analyzed in a variety of tumor subtypes as a marker of stem cells (Hermann et al., 2007; Maeda et al., 2008; Shepherd et al., 2008; Singh et al., 2003; Singh et al., 2004). Furthermore, CD133 expression in tumor cells has been shown to correlate with prognosis for patients with both oligodendroglial and astrocytic tumors (Beier et al., 2008; Zeppernick et al., 2008). This correlation might relate to differential growth characteristics seen between CD133⁺ and CD133⁻ stem cells (Beier et al., 2007).

There are few assays for accurately identifying the CD133 antigen as a stem cell marker. For instance, Hoechst 33342 staining in combination with a CD133 antibody is used to isolate CD133 positive cells via flow cytometry (Brown et al., 2007). However, this process can be labor intensive and can be particularly harsh to the cells themselves. The cells can also be directly stained with the CD133 antibody alone and assessed via flow cytometry. Although these assays both assess for protein expression, neither assay allows for direct visualization

of CD133 in the intact cell. Furthermore, glioblastoma stem cells are typically grown as neurospheres in vitro. We sought to generate a staining protocol by which we could reproducibly identify CD133 staining on the surface of glioblastoma stem cell neurospheres using established glioblastoma stem cell lines, (Lee et al., 2006; Son et al., 2009) which could also be used as an important confirmation step prior to, and in adjunct to, using flow cytometry.

2. Methods

Superfrost/plus pre-cleaned slides (Fisher Scientific, Pittsburgh, PA, USA) were coated with 100 μ l of a poly-L-ornithine and EHS laminin (Sigma, Cream Ridge, NJ, USA) coating solution in the area where the cells were to be deposited. The coating solution was allowed to set for 1 h at room temperature. The remaining coating solution was then aspirated and the slides were dried overnight at 4°C. The stem cells were plated into small dishes or flasks at approximately 50% confluency in stem cell media: neurobasal media (Gibco, North Andover, MA, USA), B27 supplement (Invitrogen, Bergisch Gladbach, Germany), N2 supplement (Gibco, North Andover, MA, USA), 0.5mM L-glutamine (Fisher Scientific, Pittsburgh, PA, USA), 50 ng/ml EGF (Upstate, Lake Placid, NY, USA), 50 ng/ml bFGF (R&D Systems, Minneapolis, MN, USA), 100 U/100 μ g penicillin/streptomycin (Gibco, North Andover, MA, USA). The stem cells were then incubated at 37 °C for 5 days or until small neurospheres formed.

For each slide, 200 μ l of media containing neurospheres in suspension was removed from the cell culture flask and FBS was added (final 10%, for increased attachment and viability). The slide clip was opened and the coated superfrost/plus pre-cleaned slide was placed followed by a single cytology funnel into the clip. The slide and funnel were secured and each slide clip was set into the Cytospin 2 centrifuge with 2 slide clip sets per sample (one slide to serve as a control). The slide clip sets were spaced appropriately to allow for even balancing. Into each funnel, 200 μ l of media was placed containing neurospheres with 10% FBS. After securing the lid, the centrifuge was run at 800 RPM for 3 min. This speed was important so that the spheres would attach sufficiently without destruction of the cell membranes and associated antigens. The slide clip was then opened and the superfrost/plus pre-cleaned slide containing the neurosphere sample was carefully removed from the funnel apparatus without disturbing the sample droplet. A circle was drawn around the sample (on the reverse of the slide) as the sample can become unidentifiable after the media has evaporated. The sample droplet was then allowed to dry for approximately 10 min at room temperature.

Fresh 0.3% w/v 1,4-benzoquinone (Sigma, Cream Ridge, NJ, USA) in 1X DPBS was then prepared. 1,4-benzoquinone is light sensitive and was prepared fresh at each use. A new sample was prepared if not used within 20 min to prevent use of a degraded sample. One hundred μ l of 0.3% w/v 1,4-benzoquinone was applied to the sample(s). As controls, we tested routine fixatives including 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), 100% ice-cold acetone (Fisher Scientific, Pittsburgh, PA, USA), and 100% ice-cold methanol (Fisher Scientific, Pittsburgh, PA, USA) via routine application of each fixative. The fixative was allowed to set on the slide(s) for 10 min. Residual fixative was then aspirated from the slide(s). The slide(s) was washed with 100 μ l of 1X PBS for 1 min and residual PBS was aspirated. Residual fixative was aspirated from the edge of the slide(s) without aspirating near the sample itself.

To make blocking buffer, 10% v/v goat serum (Invitrogen, Bergisch Gladbach, Germany) was added to Tween TBS rinsing buffer. One hundred μ l of blocking buffer was applied to the sample(s) and the buffer was allowed to incubate for 30 min at room temperature.

Blocking buffer was then added to CD133/2(293C3) pure, mouse antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) at a 1:11 dilution. One hundred μ l of primary antibody solution was applied to only one of each pair of samples and the solution was allowed to sit for 10 min at 4 °C. Residual primary antibody solution or blocking buffer was then aspirated. One hundred μ l of Tween TBS rinsing buffer was applied to the sample(s). Rinsing buffer was allowed to sit for 1 min. Residual rinsing buffer was then aspirated. This step was repeated 4 additional times.

Blocking buffer was added to Alexa Fluor 555 goat anti-mouse IgG (H + L) secondary antibody (Molecular Probes, Carlsbad, CA, USA) at a 1:1500 dilution. One hundred μ l of secondary antibody solution was applied to the sample(s) and the solution was allowed to sit for 30 min at room temperature. The second slide for each sample was stained with the secondary antibody solution alone to be used as a control. Residual antibody solution was then aspirated. One hundred μ l of Tween TBS rinsing buffer was then applied to the sample(s). Rinsing buffer was allowed to sit for 1 min and residual rinsing buffer was aspirated. This step was repeated 4 additional times. One hundred μ l of 1X PBS was applied to the sample(s). PBS was allowed to sit for 1 min and the residual PBS was aspirated.

In a separate experiment, the CD133/2(293C3) pure, mouse antibody was added to the slides and rinsed with Tween TBS as described above. Following the rinsing step, a second primary antibody, Nestin rabbit polyclonal antibody (Abcam, Cambridge, MA, USA), was added to the slide at a 1:200 dilution. One hundred μ l of primary antibody solution was applied to only one of each pair of samples and the solution was allowed to sit for 30 min at 4 °C. Residual primary antibody solution or blocking buffer was then aspirated. One hundred μ l of Tween TBS rinsing buffer was applied to the sample(s). Rinsing buffer was allowed to sit for 1 min. Residual rinsing buffer was then aspirated. This step was repeated 4 additional times.

Blocking buffer was added to Alexa Fluor 488 chicken anti-rabbit IgG (H + L) secondary antibody (Molecular Probes, Carlsbad, CA, USA) at a 1:1000 dilution. One hundred μ l of secondary antibody solution was applied to the sample(s) and the solution was allowed to sit for 30 min at room temperature. The second slide for each sample was stained with the secondary antibody solution alone to be used as a control. Residual antibody solution was then aspirated. One hundred μ l of Tween TBS rinsing buffer was then applied to the sample(s). Rinsing buffer was allowed to sit for 1 min and residual rinsing buffer was aspirated. This step was repeated 4 additional times. One hundred μ l of 1X PBS was applied to the sample(s). PBS was allowed to sit for 1 min and the residual PBS was aspirated.

Prolong Gold mounting media with DAPI (Molecular Probes, Carlsbad, CA, USA) was applied to each sample. A #0 22 $\times$ 22 mm coverslip was then mounted over the sample(s). Clear nail polish was applied to the edges of the coverslip and allowed to dry. Slide(s) were then placed in a closed container to prevent light from degrading the fluorescent signal. The slide(s) were stored in the dark at 4 °C. The slides can maintain strong fluorescent signal for up to 2 weeks.

3. Results

We found that the use of the fixative 1,4-benzoquinone generated high quality, reproducible staining for the CD133 antigen. The other routine fixatives we used did not identify consistent immunofluorescence staining for CD133 (Fig. 1). Glioblastoma stem cells exposed to the secondary antibody alone served as a control and did not display significant near red fluorescence for CD133 (Fig. 2). Glioblastoma stem cells stained with both CD133

and Nestin, an important progenitor cell marker, displayed effective reproducible staining with the 1,4-benzoquinone fixative (Fig. 3).

4. Discussion

In this report, we have established a customized staining protocol to reproducibly generate immunofluorescence staining in glioblastoma stem cell neurospheres in an effort to better characterize patient tumor specimens. In this effort we identified a novel fixative, 1,4-benzoquinone, for staining stem cell markers such as CD133, to maximize efficacy of this staining protocol by allowing better preservation of the antigen. The fixative has been previously reported to display advantages over other agents for immunofluorescence staining such as paraformaldehyde, which typically requires a higher concentration to permeabilize the cell membranes for antibody access that can potentially damage surface marker antigens (Fredens et al., 1986).

This immunofluorescence staining protocol using 1,4-benzoquinone as the fixative provides an effective and reproducible assay for identifying the CD133 marker in glioblastoma stem cells. A small number of cells are required for this assay and neurospheres do not have to be dissociated in order to produce effective staining, by using the indicated protocol. As recent data indicate that CD133⁺ cells can regain the CD133 antigen, this protocol can also prove useful in assessing the effectiveness of sorting techniques for CD133. In addition, we can analyze the presence of this antigen in stem cells grown in vitro over time (Wang et al., 2008). While there is no current literature, the methods described in this report in combination with 1,4-benzoquinone may be utilized in characterizing stem cells in other tissues.

Acknowledgments

We thank Dr. Howard Fine for his kind gift of the 0308 and 0822 glioblastoma stem cell lines. We would also like to thank Dr. Adam Goldfarb for the use of his laboratory's Cytospin 2 centrifuge.

References

- Beier D, Hau P, Proescholdt M, Lohmeier A, Wischhusen J, Oefner PJ, et al. CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Research*. 2007; 67:4010–4015. [PubMed: 17483311]
- Beier D, Wischhusen J, Dietmaier W, Hau P, Proescholdt M, Brawanski A, et al. CD133 expression and cancer stem cells predict prognosis in high-grade oligodendroglial tumors. *Brain Pathology* (Zurich Switzerland). 2008
- Brown MD, Gilmore PE, Hart CA, Samuel JD, Ramani VA, George NJ, et al. Characterization of benign and malignant prostate epithelial Hoechst 33342 side populations. *The Prostate*. 2007; 67:1384–1396. [PubMed: 17639507]
- Fredens K, Dahl R, Venge P. An immunofluorescent method for a specific demonstration of granulocytes and some of their proteins (ECP and CCP). *Histochemistry*. 1986; 84:247–250. [PubMed: 3519546]
- Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell*. 2007; 1:313–323. [PubMed: 18371365]
- Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell*. 2006; 9:391–403. [PubMed: 16697959]

- Maeda S, Shinchu H, Kurahara H, Mataka Y, Maemura K, Sato M, et al. CD133 expression is correlated with lymph node metastasis and vascular endothelial growth factor-C expression in pancreatic cancer. *British Journal of Cancer*. 2008; 98:1389–1397. [PubMed: 18349830]
- Shepherd CJ, Rizzo S, Ledaki I, Davies M, Brewer D, Attard G, et al. Expression profiling of CD133(+) and CD133(–) epithelial cells from human prostate. *The Prostate*. 2008
- Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Research*. 2003; 63:5821–5828. [PubMed: 14522905]
- Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. *Nature*. 2004; 432:396–401. [PubMed: 15549107]
- Son MJ, Woolard K, Nam DH, Lee J, Fine HA. SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma. *Cell Stem Cell*. 2009; 4:440–452. [PubMed: 19427293]
- Wang J, Sakariassen PO, Tsinkalovsky O, Immervoll H, Boe SO, Svendsen A, et al. CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. *International Journal of Cancer*. 2008; 122:761–768.
- Zeppernick F, Ahmadi R, Campos B, Dictus C, Helmke BM, Becker N, et al. Stem cell marker CD133 affects clinical outcome in glioma patients. *Clinical Cancer Research*. 2008; 14:123–129. [PubMed: 18172261]

Fig. 1.

Immunofluorescence staining for the CD133 surface antigen using glioblastoma 0308 stem cells (Lee et al., 2006). The cells were grown for 5 days in stem cell media and the staining protocol was followed as described in this report. Four fixatives were used for the purpose of identifying the ideal fixative for preserving the CD133 surface antigen. (A) The 1,4-benzoquinone fixative proved to be effective in generating reproducible staining for the CD133 surface antigen. Confocal microscopy displayed near red immunofluorescence of CD133 using the CD133/2(293C3) primary antibody and the Alexa Fluor 555 secondary antibody. The cell nucleus was identified via blue fluorescence using DAPI. (B–D) Glioblastoma 0308 neurospheres were fixed with 4% paraformaldehyde (B), 100% ice-cold

acetone (C), and 100% ice-cold methanol (D). The neurospheres were then stained with the CD133/2(293C3) primary antibody and the Alexa Fluor 555 secondary antibody. No significant near red immunofluorescence staining was identified.

Fig. 2.

Immunofluorescence staining for the CD133 surface antigen using the 1,4-benzoquinone fixative. (A and C) Glioblastoma 0308 (A) and 0822 (C) stem cells were stained with the CD133/2(293C3) primary antibody and the Alexa Fluor 555 secondary antibody. Immunofluorescence was assessed via confocal microscopy. Immunofluorescence for the cell nucleus (DAPI-blue) and for CD133 (AF555-red) was seen with co-localization. (B and D) Glioblastoma 0308 (B) and 0822 (D) stem cells were also stained with the Alexa Fluor 555 secondary antibody alone as a control and did not display near red immunofluorescence for CD133.

Fig. 3. Immunofluorescence staining of glioblastoma 0308 stem cells with the CD133/2(293C3) primary antibody and the Alexa Fluor 555 secondary antibody using the 1,4-benzoquinone fixative. (A) Glioblastoma 0308 stem cells were stained with the CD133/2(293C3) primary antibody and the Alexa Fluor 555 secondary antibody. (B) Glioblastoma 0308 stem cells were stained with the Nestin primary antibody and the Alexa Fluor 488 secondary antibody. (C) The cell nucleus was identified via blue fluorescence using DAPI. (D) Dual immunofluorescence staining for CD133 and Nestin was seen with co-localization (A).