Flow cytometric analysis of mouse neurospheres based on the expression level of RANDAM-2

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Abstract

RANDAM-2, a type-I transmembrane antigen constitutively expressed on the neuronal cell lineage during mouse neurogenesis, shows the highest expression level between embryonic day 8.5 (E8.5) and E10.5. As the period well overlaps with the proliferating stages of neural stem cells (NSCs), it is conceivable that NSCs are efficiently separable based on the expression level of RANDAM-2. In this paper, we show that NSCs can be efficiently enriched as RANDAM-2high+ cells by fluorescence-activated cell sorting. Many cells in the RANDAM-2high+ cells had the characteristics of the self-renewal capability and potential for multilineage differentiation into neural cells. In contrast, almost all of the RANDAM-2low−/− cells exhibited not only the extremely low self-renewability but the differentiation capability restricted to neurons. These two subpopulations also differed from each other in terms of the expression level of molecules associated with neural differentiation. These findings demonstrate that RANDAM-2 can be regarded as a useful marker for enrichment of NSCs.

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The expression pattern of RANDAM-2, a sialomucin-like type-I transmembrane glycoprotein expressed on the cells of neuronal cell lineage during mouse embryogenesis [7,8], is quite intriguing, because it gives the highest expression level between the embryonic stage 8.5 days (E8.5) and E10.5, and because its expression level in undifferentiated cells is far higher than that in the differentiated neurons [7]. The period in rodent embryogenesis corresponds to the proliferating stages of neural stem cells (NSCs) in central nervous system (CNS) [4]. On the other hand, although the physiological aspects of RANDAM-2 are still unknown, it is expected that RANDAM-2 is concerned with cell growth, because RANDAM-2 and its homologs are commonly highly expressed on the proliferating cells [8,11,16]. From these findings, it is reasonable to speculate that RANDAM-2 is an antigen highly expressed on the undifferentiated cells such as NSCs in CNS. Therefore, we examined the expression levels of RANDAM-2 in neurospheres derived from CNS, and analyzed further about the capabilities of self-renewal and multilineage neural differentiation of the cells sorted based on the expression level of RANDAM-2.

Neurospheres were generated as previously described [8]. Briefly, mouse E13.5 telencephalon were dissociated into
single cells by trypsin-EDTA treatment, and the cells were plated on a 60-mm culture dish with the neurosphere culture medium, F12/DMEM medium comprising 5 mM HEPES, 2 mM L-glutamine, 30 mM sodium selenite, 60 μM putrescine, 20 nM progesterone, 3 mM sodium bicarbonate, 25 μg/ml insulin, 100 μg/ml transferrin, and 0.6% glucose, containing 20 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF). The neurospheres obtained by this culture were defined as first neurospheres in this study. The subculture of neurospheres was performed at 6–8-day intervals as follows: The neurospheres were dissociated into single cell suspension by mechanical pipetting, and the cells were plated onto a new dish with the neurosphere culture medium containing growth factors as described above.

Neurosphere membrane lysates were prepared as described previously [8]. The lysates (10 μg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 4–20% acrylamide gradient gel followed by electrotransfer onto a polyvinylidene difluoride membrane. The transferred membranes were incubated with SKY-2 monoclonal antibody (mAb) [7], specific to RANDAM-2. After washing, the membrane was incubated with peroxidase-conjugated anti-rat IgG F(ab′)2, followed by visualization with a chemiluminescence detection system (Amersham Biotech).

Flow cytometric analysis and cell sorting by fluorescence-activated cell sorting (FACS) were performed as follows: the single cell suspension of neurospheres (5 × 10⁵ cells per one analysis) was incubated with SKY-2 mAb, phycoerythrin (PE)-conjugated mCD24 mAb (PE-mCD24 mAb; BD Bioscience), specific to mCD24 to be one of cell surface markers on haematopoietic stem cells (HSCs) [5], or biotinylated PNA (Bio-PNA) (Seikagaku Corporation), reactive to a carbohydrate antigen (Galβ1-3GalNAc) [15], washed, and then incubated with FITC-conjugated anti-rat IgG F(ab′)2 or streptavidin-allophycocyanin (ST-APC; BD Bioscience). The stained cells were analyzed by a FACS Aria (BD Bioscience). The dead and aggregated cells were judged for elimination by propidium iodide staining (BD Bioscience) and microscopy, respectively. In triple color immunofluorescence analysis and cell sorting, 5 × 10⁶ cells were simultaneously incubated with SKY-2 mAb, PE-mCD24 mAb, and Bio-PNA, washed, and then incubated with FITC-conjugated and ST-APC. The stained cells were analyzed by FACS and sorted into neurosphere culture medium without growth factors, and the sorted cells were counted and immediately used for analysis. The cell size in scatter analysis was determined based on the size of standard particles (10 μm in diameter) (Beckman Coulter).

The self-renewal capability of the FACS-sorted cells was estimated by neurosphere forming assay. The sorted cells (2 × 10³ cells per well on a 24-well culture plate) were cultured for 6 days in the neurosphere culture medium containing bFGF and EGF. On the last day of the culture, the number of neurospheres clonally expanded from the cells was counted. The number of neurospheres counted was regarded as the self-renewability of NSC in this study. For neural differentiation assay, the neurospheres generated from the sorted cells were cultured for 3 days in the neurosphere culture medium without any growth factors in a 2-well chamber slide precoated with 0.2% polyethyleneimine in PBS, and the cells were performed the triple color staining with MAP2 mAb (Sigma), glial fibrillary acidic protein mAb (GFAP mAb; PROGEN), and O4 mAb (American Type Culture Collection), specific to neurons, astrocytes and oligodendrocytes, followed by incubation with Cy5-conjugated anti-mouse IgG F(ab′)2, FITC-conjugated anti-mouse IgG F(ab′)2 and Cy3-conjugated anti-mouse IgM. The stained cells were examined under a microscope (Axiovert 100 M; Carl Zeiss) equipped with a confocal laser scanning system (LSM510; Carl Zeiss). The numbers of the total cells, MAP-2-positive cells, GFAP-positive cells, O4-positive cells and the cells having no reactivity to the mAbs were counted on the photographs obtained as phase contrast and fluorescence images of the cells with the microscope described above.

Total RNA was isolated from neurosphere pellets with an extraction kit (RNeasy; QIAGEN). RT-PCR was performed with KOD polymerase (Toyobo) with a 1 μl template. The glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was used as an internal standard for mRNA quantification by PCR with the sense (5′ ACCACGTCTCATGCCCATC 3′) and antisense (5′ TCCACCACCCTGTTGCTGTA 3′) primers from Clontech (Palo Alto).

First of all, the single cell suspension of neurospheres prepared by mechanical pipetting was subjected to scatter analysis based on the cell size with side scatter (SSC) and forward scatter (FSC) to obtain the appropriate scatter gating for the analysis of the neurosphere-derived cells. The “a” scatter gating exclusively gave single viable cells, and the cell size in the “a” scatter gating was about 5–15 μm in diameter.

The cells in “a” gating in the neurosphere-derived cells triple color-stained with SKY-2 and mCD24 mAbs and Bio-PNA were subjected to FACS analysis for the sorting of cells. The cells were separated into two subpopulations, R1 (red color) and R2 (blue color), based on the expression level of RANDAM-2, resulting that the cell surface antigen phenotypes of the R1 and R2 cells were RANDAM-2high mCD24high PNA− and RANDAM-2low mCD24low PNA−, respectively (Fig. 1A), and that the cell number ratios of R1 and R2 cells in “a” gating were 4.1% and 4.6%, respectively (Fig. 1B). As expected, the R1 and R2 cells expressed RANDAM-2 in the membrane fraction quite strongly and weakly, respectively (Fig. 1C). Interestingly, the R1 cells in “a” gating have comparatively larger sizes from 8 to 15 μm, whereas the R2 cells showed relatively smaller sizes from 5 to 10 μm (Fig. 1A).

The R1 and R2 cells were examined for their self-renewability. As shown in Fig. 2A, the R1 cells formed neurospheres (R1 neurospheres) at 38.7%, whereas the R2 cells gave the formation ratio at 8.3% (R2 neurospheres). The formation ratio of the presorting cells was 14.5%. In addition, when the neurospheres were classified based on the diameter, roughly reflecting the self-renewal capability [6], the formation ratio of larger neurospheres (>50 μm in diameter) in the R1 cells was 20.2%, whereas those in the presorting and R2 cells were 6.4% and 2.7%, respectively (Fig. 2A). These results indicate that there is a clear difference between the R1 and R2 cells in the self-renewal capability. The R1 and R2 neurospheres collected were...
dissociated by pipetting, and the cells were again subjected to neurosphere forming assay, resulting that the neurosphere forming ratio of the second R1 cells derived from the R1 neurospheres was 54.9%, whereas that of the second R2 cells from the R2 neurospheres was only 2.4% (Fig. 2B). There was no significant change in the ratio between the presorting (14.5%) and second presorting cells (Fig. 2B). When the comparison was carried out based on the size of neurospheres (>100 μm in diameter), the second R1 cells gave the formation ratio at 29.4%, whereas no neurosphere was formed from the second R2 cells (Fig. 2B). Thus, the difference of self-renewal capability between the R1 and R2 cells was quite evident. These results indicate that the cells such as NSCs having the self-renewal capability can be enriched as RANDAM-2high+ ones.

The second R1 and R2 neurospheres were further analyzed for the capability of neural differentiation into neurons, astrocytes, and oligodendrocytes. The cells differentiated from the second R1 neurospheres indeed showed the multiple differentiation into neurons, astrocytes, and oligodendrocytes as revealed by the specific mAbs (Fig. 3A). Although these antigens sometimes seemed to be simultaneously expressed on the same cells (Fig. 3A), most of them were simply caused by overlapping of the positive cells each other. The second presorting cell-derived neurospheres had fundamentally the similar capability to that of the second R1 neurospheres (Fig. 3A). On the other hand, most of the cells differentiated from the second R2 neurospheres were observed as MAP2 mAb-positive (Fig. 3A). The qualitative data in Fig. 3A was converted quantitatively by counting the number of each differentiated cell (Fig. 3B). The second R1 neurospheres gave rise to multiple neural cell lineages including neurons (~36%), astrocytes (~39%), and oligodendrocytes (~10%), whereas the differentiation of the second R2 neurospheres was restricted almost exclusively to neurons (~80%).

The present data strongly suggested that the second R1 and R2 cells enrich as NSCs and the neuronal differentiating cells. This was further analyzed by RT-PCR for detecting the cDNAs associated with NSCs and neural differentiation. The Sox1 [12], Musashi1 [14], and MCM2 [17], which are the selective markers of NSCs, were certainly expressed in the second R1 neurospheres, whereas their expression in the second R2 neurospheres...
Fig. 2. Self-renewability of R1 and R2 cells. (A) The neurosphere formation ratio of R1 cells (38.7%) showed approximately five-fold higher than that of R2 cells (8.1%). In addition, about 20% of R1 cells formed as larger neurospheres (>50 μm in diameter), whereas R2 cells gave the larger spheres at only 2.7%. The values were represented as percentage (mean ± S.D.) of neurosphere formation from three independent experiments. Bars in A and B, 50 μm. (B) The second R1 and R2 cells derived from the first R1 and R2 neurospheres were analyzed by neurosphere formation assay. The second R1 cells gave a formation capability of neurospheres at 54.9%. In contrast, only 2.4% was obtained from the second R2 cells. Note that no large neurosphere (>100 μm in diameter) was formed from R2 cells.

was weak or negligible (Fig. 3C). This was also the case for the expressions of Hes1 and Hes5 [2] (Fig. 3C). In contrast, the expression of MATH3 and NeuroD [1] did not show such a remarkable difference between the second R1 and R2 neurospheres (Fig. 3C). These results well supported the results of Fig. 2 and Fig. 3A and B.

In the present study, we showed that RANDAM-2 is a useful cell surface antigen for enrichment of NSCs by FACS analysis. About 55% of cells in the second R1 cells (RANDAM-2\textsuperscript{high+} mCD24\textsuperscript{high+} PNA\textsuperscript{−}) formed neurospheres (second R1 neurospheres) (Fig. 2B), and the second R1 neurospheres really differentiated into three neural cell lineages of neurons, astrocytes, and oligodendrocytes (Fig. 3A and B). In contrast, the neurosphere formation capability of the second R2 cells (RANDAM-2\textsuperscript{low−}/mCD24\textsuperscript{low−} PNA\textsuperscript{−}) was very low (Fig. 2B), and the second R2 neurospheres differentiated almost exclusively into neurons (∼80%) (Fig. 3A and B). These experimental results were also supported by the analysis of the selective markers for NSCs such as Sox1 [12], Musashi1 [14], and MCM2 [17] along with that of the transcription factors having a repressor-type bHLH motif [1] in the second R1 and R2 neurospheres (Fig. 3C).

The scatter analysis is nevertheless essential for obtaining the optimal scatter gating of the cells for FACS analysis [10,13]. The cell size (8–15 μm) by scatter analysis of RANDAM-2\textsuperscript{high+} cells (R1 cells) having a self-renewable potency in “a” gating was roughly similar to that (>12 μm and ∼20 μm) reported by Reitze et al. [13] and Murayama et al. [10]. In FACS analysis of mouse neurospheres, almost all of the cells in “a” gating showed the cell surface antigen phenotype of RANDAM-2\textsuperscript{+} mCD24\textsuperscript{+} PNA\textsuperscript{−} (Fig. 1). Rietze et al. [13], however, reported the presence of four subpopulations, mCD24\textsuperscript{low+} PNA\textsuperscript{high+}, mCD24\textsuperscript{midle/high+} PNA\textsuperscript{high+}, mCD24\textsuperscript{midle/high+} PNA\textsuperscript{low+}, and mCD24\textsuperscript{low+} PNA\textsuperscript{low+}, in adult mouse CNS-derived neurospheres. In our study, however, we were not able to find any PNA\textsuperscript{high+} cell population in the dissociated cells from neurospheres. It is uncertain why such differences arose, but might be conceivable that the method for obtaining single cells from neurospheres may result from differing each other. Generally, the analysis of physiological expression pattern of membrane surface antigens on cells by FACS should exclude the pretreatment of the analyzing cells with proteases, because the expression pattern of membrane surface antigens can be seriously altered. As we adopted mechanical pipetting, our FACS data seems to more physiologically reflect the expression levels of PNA on the single viable cells from neurospheres.
In our study, about 55% of R1 cells (RANDAM-2^{high+} mCD24^{high+} PNA^{-}) were strongly suggested to correspond to NSCs based on the assays of self-renewability and neural differentiation. On the other hand, Rietze et al. [13] reported that NSCs in mouse adult brain are found as the CD24^{low+} PNA^{low+} cells. Thus, the expression level of mCD24 is disagreement between our results and the results by Rietze et al. [13]. Our result concerning the expression level of mCD24 seems to correspond to the case of the immune system, in which mCD24 is highly expressed in the proliferative lymphocytes [3,9]. Although we do not have the data to explain the reason why such a discrepancy arose, it might be possible to be attributed to the method obtaining single cell suspension of neurospheres as described above.

In order to prove more evidently that RANDAM-2 is really a selective marker for NSCs, the sorted cells as RANDAM-2^{high+} is required to show their own multiple differentiation potency into neural cells in vivo.

References


