Characterization of a monoclonal antibody, Namu mAb, which reacts to the subventricular zone in mouse brain

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The lateral ventricle in adult mammalian brain is widely acknowledged as one of the areas that undifferentiated neural cells such as neural stem cells and neural progenitor cells inhabit. However, immunological aspects of neural stem cells in the lateral ventricle are still under debate. Here, we report the generation and characterization of a novel monoclonal antibody (mAb), called Namu mAb, which stains the subventricular zone in the lateral ventricle of adult mouse brain. Namu mAb reacted to the cells in the subventricular zone and never reacted to differentiated neural cells such as neurons and glial cells such as astrocytes and oligodendrocytes. Its reaction pattern for the subventricular zone and the neurospheres was similar to that of Nestin and glial fibrillary acidic protein mAbs. Namu mAb recognition molecule, Namu antigen, was a 50 kDa protein present in the cytoplasmic fraction of mouse brain, and its expression was clearly observed in neurospheres cultured in the presence of epidermal growth factor, but it was never or only weakly induced in the presence of basic fibroblast growth factor or leukemia inhibitory factor. Collectively, it is concluded that Namu mAb specifically reacts to undifferentiated neural cells in mouse brain. ***NeuroReport*** **00:000–000**

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Introduction

The lateral ventricle in adult mammalian brain consists of two cell layers, the ventricular zone and the subventricular zone, which are contiguous to each other. It was reported that the cells in the dorsal side of the lateral ventricle in adult brain had self-renewal capacity and multipotency, by which undifferentiated neural cells such as neural stem cells (NSCs) and/or neural progenitor cells (NPCs) are characterized [1]. Although the existence of NSCs in the lateral ventricle of adult brain is uncontroversial, the immunological aspects of the NSCs have not yet been clarified. One of the reasons for this is that there are few effective biomarkers for the identification of NSCs. We attempted to search for a novel biomarker effective for the immunological identification of NSCs in the lateral ventricle.

Materials and methods

Animals

Female F344 rats, female BALB/c mice, pregnant ICR mice, and 8-week-old ICR mice were purchased from Charles River Laboratories (Tokyo, Japan). All the animals were housed in the Ohu University Animal Care Facility and the experiments were performed according to the guidelines of Ohu University Animal Research Committee.

Cells and cell cultures

Mouse myeloma (PAI) cells were cultured in RPMI-1640 containing 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid), 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 10% fetal calf serum. Hybridoma cells were cultured in the culture media, to which hypoxanthine, aminopterin, and thymidine (Invitrogen, Carlsbad, California, USA) were added for PAI cells. Neurospheres were prepared according to the previous method [2].

Generation of monoclonal antibodies

The immunogen used in this experiment was prepared as follows: the periventricular area of 8-week-old mouse brains was fixed with 4% paraformaldehyde–PBS was homogenized in two volumes of 25 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 μM aprotinin, and 1 mM iodoacetoamide. The homogenized samples were stored at −80°C until use. Female Wistar rats, 3-week old, were immunized three times in the footpads at a 5-day interval with 100 μl immunogen per footpad prepared by mixing the same volume of immunogen (100 μg/ml) and a complete adjuvant, Titer Max Gold (CytRx Corporation, Norcross, Georgia, USA). Three days after the final booster immunization, popliteal lymph node cells were fused with PAI

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myeloma cells using polyethylene glycol 1500 (Roche Diagnostic GmbH, Mannheim, Germany). Hybridoma cells were screened and cloned by immunohistochemical analysis as described below.

Immunohistochemical and immunocytochemical staining

The immunohistochemical and immunocytochemical stainings were performed according to the previous methods [3]. The antibodies used were CD133 (Chemicon, Temecula, California, USA), Nestin (Chemicon), GFAP (Stermberger, Berkley, California, USA), neurofilament (NF) 200 (Sigma, St Louis, Missouri, USA), and Rip (University Iowa, Iowa City, Iowa, USA). The second antibodies used were FITC-conjugated anti-rat IgG (Jackson ImmunoResearch, West Grove, Pennsylvania, USA), Cy3-conjugated anti-rat IgG (Jackson ImmunoResearch), and Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch).

Western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed according to the previous methods [3]. In brief, the samples (2 μg/lane) boiled under reducing conditions were separated by SDS-PAGE in a 4–20% gradient gel and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon: Nippon Millipore, Tokyo, Japan). The blotted membrane was blocked with 5% skim milk in PBS, followed by incubation with the hybridoma culture supernatant for 45 min, and incubated with peroxidase-conjugated anti-rat IgG (2500-fold diluted; Jackson ImmunoResearch). The antibody-reacted bands were visualized using a chemiluminescent detection system (ECL; GE Healthcare, Buckinghamshire, UK). For metaperiodate oxidation of the antigen, the PVDF membranes electroblotted with the cytoplasmic fraction were treated with or without 25 mM sodium periodate ($NaIO_4$) in 100 mM acetate buffer, pH 4.0, for 30 min at room temperature in the dark. The membranes washed with PBS were subjected to immunological detection as described above.

Namu antigen expression assay in neurosphere cells

A single-cell suspension of neurospheres was prepared by mechanical pipetting and plated onto a 12-well plate in 5×10^4 cells/well. The cells were cultured for 8 days in culture medium for neurospheres with different combinations of three growth factors: epidermal growth factor (EGF), basic fibroblast growth factor, and leukemia inhibitory factor (LIF). Four days after the culture was initiated, half the amount of these culture media was changed. On day 8, the cells were collected and used for immunocytochemical analysis.

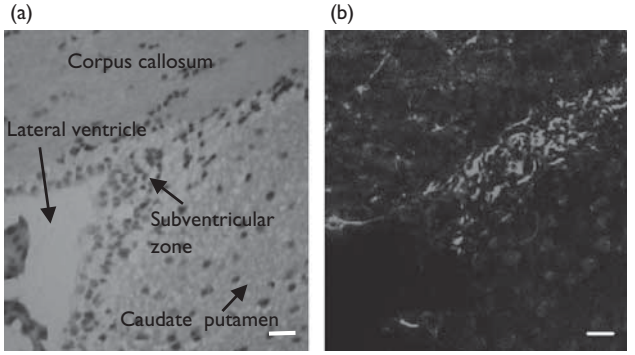
Results

One type of the hybridoma cells raised against the immunogen was produced a monoclonal antibody (mAb) which had the reactivity against specific cells in the lateral ventricle of adult mouse brain. We designated the mAb as Namu mAb. Because Namu mAb clearly and streakily reacted to the subventricular zone cells in the lateral ventricle (Fig. 1), we focused on its reactivity in the subventricular zone and performed double immunostaining to compare the immunoreactivity of Namu mAb and other mAbs against undifferentiated and differentiated neural cell biomarkers. A relatively large number of Namu mAb-positive ($Namu^+$) $Nestin^+$ and $Namu^+$ $GFAP^+$ cells were found in the subventricular zone, whereas $Namu^+$ $CD133^+$ cells were hardly observed and neither NF200 nor Rip expression was observed among $Namu^+$ cells (Fig. 2a). In addition, Namu mAb never reacted to the $GFAP^+$ astrocytes inhabiting cerebric areas such as the cerebral cortex, putamen, and corpus callosum (Fig. 2a). We further examined the immunoreactivity of Namu mAb using neurospheres, which are spherical populations of heterogeneous cells that consist of undifferentiated neural cells such as NSCs, NPCs, and neuroblasts [4]. The majority of cells in the neurospheres cultured in the presence of growth factors were $Namu^+$ $Nestin^+$, although a few $Namu^+$ $CD133^+$ and $Namu^+$ $GFAP^+$ cells were also observed (Fig. 2b). Namu mAb did not react to the neurons and glial cells that had migrated and differentiated from neurospheres (Fig. 2c). Although Namu mAb appears to have reacted to some of the nonmigrated $NF200^+$ or $GFAP^+$ cells (Fig. 2c), these cells may be in the process of differentiation.

Next, we carried out biochemical characterization of the Namu mAb recognition molecule. Samples of the crude membrane fraction and the cytosol fraction prepared from the periventricular area of 8-week-old brains were applied to SDS-PAGE under reducing conditions, followed by western blotting. A single molecular weight band, designated as Namu antigen, was detected at 50 kDa in the cytosol fraction, but not in the crude membrane fraction (Fig. 3a). To determine whether Namu antigen is expressed in other areas in 8-week-old brain, the cytosol fractions prepared from the caudate putamen and cortex areas were analyzed by western blotting. Namu antigen was undetectable in these samples (Fig. 3b). In addition, it was also undetectable in organs of adult mice other than 8-week-old brain (Fig. 3b). Then, we analyzed the Namu mAb recognition epitope on Namu antigen. The reactivity of Namu mAb to Namu antigen was not lost after the treatment with $NaIO_4$, but rather it seemed to have been enhanced (Fig. 3c). This result indicates that Namu mAb reacts to the core polypeptide of Namu antigen.

Finally, we attempted to identify the growth factors involved in the expression of Namu antigen using neurospheres. Neurospheres cultured in the presence of EGF clearly reacted to Namu mAb, whereas those with basic fibroblast growth factor and leukemia inhibitory factor were almost and completely nonreactive, respectively

Fig. 1



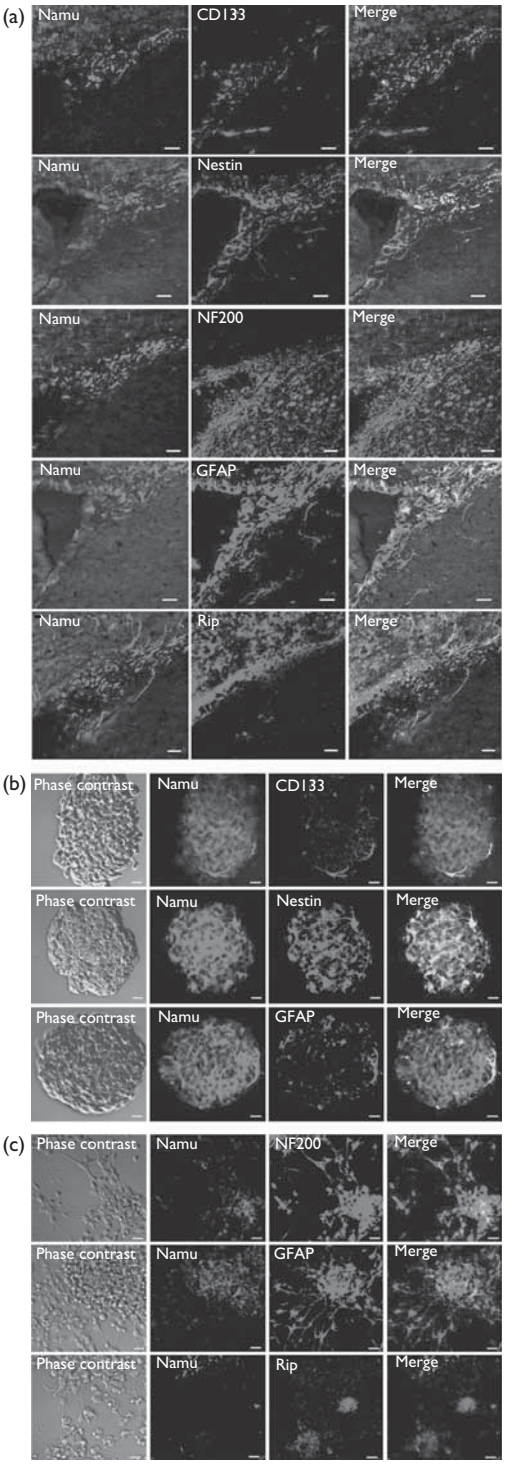
Immunohistochemical staining of adult mouse LV by Namu mAb. Frozen sections (coronal sections) were fixed and stained with Namu mAb (b) or Hematoxylin–Eosin (a). Namu mAb streakily stained the subventricular zone cells. Scale bars = 20 μm. LV, lateral ventricle.

(Fig. 4). These results suggest that the expression of Namu antigen is induced by EGF.

Discussion

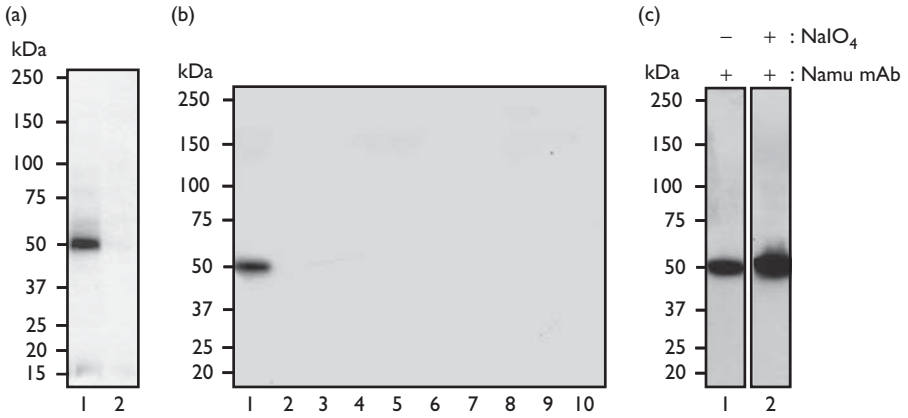
Namu mAb specifically reacted to the cells in the subventricular zone of adult mouse brain and neurospheres, but it never reacted to differentiated neural cells such as neurons and glial cells. Namu antigen was a soluble protein in the cytoplasm with a molecular weight of 50 kDa and restrictedly expressed in the periventricular area of mouse brain and its expression was regulated by EGF signals. The immunoreactivity of Namu mAb against subventricular zone cells and neurospheres was similar to that of Nestin and GFAP mAbs. Many Namu$^+$ cells in the subventricular zone and neurospheres were Nestin$^+$ and GFAP$^+$ (Fig. 2). However, the immunological characterizations of these two common mAbs and Namu mAb clearly differed in some respects. First, the molecular weights of the recognition molecules of Namu and Nestin mAbs differ significantly from each other [5]. Second, Nestin is an intermediate filament [5,6] whereas Namu antigen was a soluble protein in the cytoplasm. Although the molecular weight of GFAP [7] is similar to that of Namu antigen, GFAP is also an intermediate filament like Nestin [8] unlike Namu antigen. Third, the expression of Namu antigen was regulated by EGF signals (Fig. 4), whereas neither Nestin nor GFAP is expressed by this regulation. Recently, it was reported that activated NSCs and quiescent NSCs in the lateral ventricular zone were GFAP$^+$ EGFR$^+$ and GFAP$^+$ EGFR$^-$, respectively [9]. In this experiment, it was not possible to decide whether EGFR is expressed on Namu$^+$ cells because the immunohistochemical detection of EGFR is very difficult [10]. However, as the expression of Namu antigen was induced by EGF (Fig. 4), it is reasonable to consider that Namu$^+$ cells have EGFR. If this idea is on the right track, it is quite plausible that Namu$^+$ GFAP$^+$ cells observed in this experiment correspond to activated NSCs.

Fig. 2



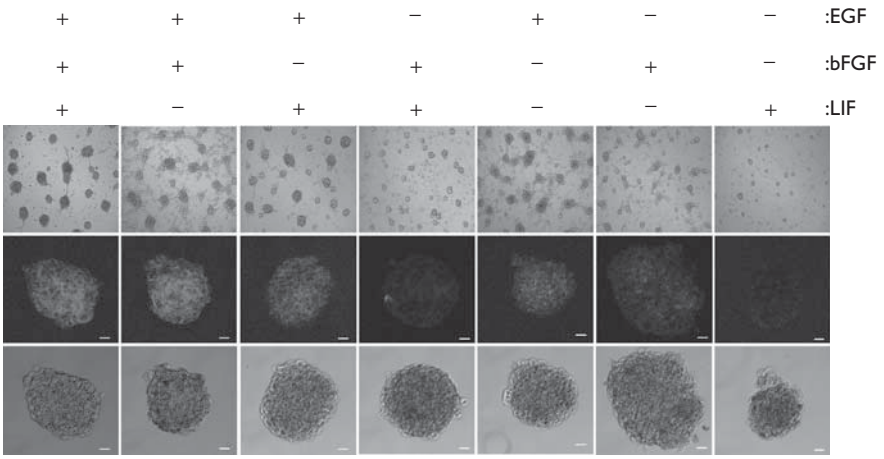
Immunoreactivity of Namu mAb in the subventricular zone and neurospheres. (a) Double immunostaining of fixed-frozen sections (coronal sections) was carried out by Namu mAb (green) and CD133 mAb (red), Nestin mAb (red), NF200 mAb (red), GFAP mAb (red), or Rip mAb (red). Scale bars = 20 μm. (b) Double immunostaining of fixed neurospheres was carried out by Namu mAb (green) and CD133 mAb (red), Nestin mAb (red), or GFAP mAb (red). Scale bars = 20 μm. (c) Double immunostaining of neural cells differentiated from neurospheres was carried out by Namu mAb (green) and NF200 mAb (red), GFAP mAb (red), or Rip mAb (red). Scale bars = 20 μm.

Fig. 3



Specificity of Namu mAb. (a) Biochemical identification of the Namu mAb recognition molecule. Cytoplasmic fraction (lane 1) and membrane fraction (lane 2) prepared from the periventricular area of 8-week-old brain were separated by SDS-PAGE, followed by western blotting with Namu mAb. (b) Expression of Namu antigen in the brain and other organs of 8-week-old mice. Cytoplasmic fractions prepared from the periventricular area (lane 1), caudate putamen area (lane 2), cortex area (lane 3), cerebellum (lane 4), lung (lane 5), heart (lane 6), liver (lane 7), kidney (lane 8), spleen (lane 9), and testis (lane 10) were subjected to western blotting with Namu mAb. (c) Recognition epitope analysis on Namu antigen. The cytoplasmic fraction of the periventricular area of 8-week-old brains was separated by SDS-PAGE and electroblotted onto PVDF membranes. The blots were treated with (lane 2) or without (lane 1) $NaIO_4$, followed by incubation with Namu mAb (lanes 1 and 2). PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

Fig. 4



Relation between the expression of Namu antigen and the growth factors. Various combinations of growth factors were added to the culture medium for neurospheres. On the eighth day, each neurosphere was fixed and stained with Namu mAb (green). bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; LIF, leukemia inhibitory factor.

Conclusion

Our study suggests that Namu mAb is a novel mAb that reacts specifically to undifferentiated neural cells in the subventricular zone in adult mouse brain, although final confirmation awaits gene cloning of its recognition molecule (Namu antigen). It was also suggested that $Namu^+$ cells are $EGFR^+$ and thus have self-renewal capacity because Namu antigen was expressed in the neurospheres cultured in the presence of EGF. This specificity of Namu mAb may be useful for the immunological identification of NSCs and/or NPCs in adult mouse brain.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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