Leptomeningeal-Derived Doublecortin-Expressing Cells in Poststroke Brain

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Increasing evidence indicates that neural stem/progenitor cells (NSPCs) reside in many regions of the central nervous system (CNS), including the subventricular zone (SVZ) of the lateral ventricle, subgranular zone of the hippocampal dentate gyrus, cortex, striatum, and spinal cord. Using a murine model of cortical infarction, we recently demonstrated that the leptomeninges (pia mater), which cover the entire cortex, also exhibit NSPC activity in response to ischemia. Pia-ischemia-induced NSPCs expressed NSPC markers such as nestin, formed neurosphere-like cell clusters with self-renewal activity, and differentiated into neurons, astrocytes, and oligodendrocytes, although they were not identical to previously reported NSPCs, such as SVZ astrocytes, ependymal cells, oligodendrocyte precursor cells, and reactive astrocytes. In this study, we showed that leptomeningeal cells in the poststroke brain express the immature neuronal marker doublecortin as well as nestin. We also showed that these cells can migrate into the poststroke cortex. Thus, the leptomeninges may participate in CNS repair in response to brain injury.

Introduction

USING A MURINE MODEL of cortical infarction [1], we recently demonstrated that the leptomeninges (pia mater), which cover the entire cortex, exhibit neural stem/progenitor cell (NSPC) activity in response to ischemia in adult brains [2]. Pia-ischemia-induced NSPCs (iNSPCs) expressed the NSPC marker nestin, formed neurosphere-like cell clusters with self-renewal ability, and differentiated into neurons, astrocytes, and oligodendrocytes [2], indicating that they have stem cell capacity similar to other NSPC types. However, we demonstrated that iNSPCs were not completely identical to previously described NSPCs [2], including subventricular zone astrocytes [3], ependymal cells [4], reactive astrocytes [5], resident glial cells [6], and oligodendrocyte precursor cells [7]. At almost the same time [2], Decimo et al. showed that spinal cord meninges were potential sources of stem/progenitor cells that undergo neuronal differentiation after injury [8]. These findings suggest that the leptomeninges that cover the entire central nervous system (CNS), including the brain [2] and spinal cord [8], may have a common repair system in response to injuries. To address this question, we labeled the pial cells using a green fluorescent protein (GFP) expression vector [1] and investigated their potential contribution to cortical neurogenesis in the poststroke brain.

Mice were sacrificed on day 3 after stroke and immunohistochemistry was performed [1,2,9,10]. Consistent with previous findings [2], cells in the poststroke leptomeninges expressed NSPC markers, such as nestin and vimentin (Fig. 1A–E). In an adherent monolayer culture [2,9], most pial cells isolated from the poststroke leptomeninges expressed nestin [2], while the number of nestin-positive cells coexpressing the immature neuronal cell marker doublecortin (DCX) increased under conditions conducive to differentiation [2,10] (Fig. 1F–H). DCX expression was also confirmed by reverse transcription–polymerase chain reaction (RT-PCR) (Fig. 1I) and western blot analysis (~45 kDa) (Fig. 1J). In the poststroke brain, DCX-positive cells were observed within the poststroke pia mater and cortex where they appeared to localize near nestin-positive cells (Fig. 2A); however, they were not observed in the nonischemic ipsilateral pia mater and cortex (Fig. 2B). To trace pial cells after brain injury, we labeled the leptomeninges in the left middle cerebral artery (MCA) area using a GFP-expressing vector 2 days before stroke (Fig. 2C, D). In the absence of ischemia, ~38.3% ± 12.5% GFP-positive leptomeningeal cells were observed in the MCA area and they were observed outside the laminin-positive cells [8], indicating that they were localized within the pia mater but not in cortical layer 1 (Fig. 2E). These GFP-positive cells did not express nestin and DCX at that time (data not shown), which

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FIG. 1. Immunohistochemistry (A) showed that nestin- and vimentin-positive cells were present in the poststroke pia mater and cortex (nestin: B, D, E, green; vimentin: C–E, red; DAPI: B–E, blue). Three days after plating in the cell-differentiation-inducing medium, some of the nestin-positive cells isolated from the poststroke pia mater coexpressed DCX (nestin: F, H, green; DCX: G, H, red; DAPI: F–H, blue). DCX-positive cells were also confirmed by RT-PCR (I) and western blot (J) analyses. Scale bar: 100 μ m (B) and 50 μ m (E and F). Results shown are representative of 5 replicates of the experimental protocol. DAPI, 4',6-diamino-2-phenylindole; DCX, doublecortin; L1, cortical layer 1; P, pia mater; RT-PCR, reverse transcription–polymerase chain reaction.

agreed with reports that nestin- [2] and DCX-positive cells (Fig. 2B) are not found in the leptomeninges in the absence of ischemia. However, some GFP-labeled pial cells within the poststroke area coexpressed nestin (Fig. 2F) and DCX (Fig. 2G) on day 3 after stroke, and they migrated to the poststroke cortex (Fig. 2F, G). Although GFP-positive cells were also observed in the nonischemic ipsilateral leptomeninges (<10%) on day 3 after stroke, they did not express nestin and DCX (data not shown), which agreed with a study that observed nestin-positive cells in the ischemic pia mater but not nonischemic pia mater [2]. These results indicate that in response to ischemia, pial cells generate nestin-positive cells that were presumably iNSPCs [2] and DCX-positive immature neurons in the poststroke cortex, indicating their potential contribution to cortical regeneration. However, the number of DCX-positive cells in the poststroke pia mater and cortex gradually decreased at subsequent time points (Fig. 2H), which was consistent with the number of nestin-positive iNSPCs [2]. Thus, to further investigate the potential contribution of leptomeningeal cells to cortical neurogenesis in the poststroke brain, we obtained brain slices 3 days after stroke and incubated them for 7 days in a medium containing basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), as described previously [2], which promoted the proliferation of pial iNSPCs. DCX-positive cells were not observed in the nonischemic ipsilateral pia mater and cortex (Fig. 2I), but numerous cells were observed in the poststroke pia mater and cortex (Fig. 2J) together with an increased number of nestin-positive cells (Fig. 2K–M).

In the present study, we found that DCX-positive cells were developed in the poststroke pia mater and cortex as well as nestin-positive cells [2], although they were not observed in the nonischemic ipsilateral pia mater and cortex. These findings suggest that ischemia is essential for the induction of NSPCs and/or neuronal progenitors in these regions. In developing embryos, DCX was initially expressed

in the outer cortical region preplate, which is covered by pia during the early stages, and this expression pattern extended into the intermediate zone during the later stages [11]. As with the developmental findings, DCX-positive immature neurons were also observed in response to ischemia, predominantly in the cortical surface adjacent to the injured regions, that is, the poststroke pia mater rather than the poststroke cortex. We also demonstrated *in vivo* generation of DCX-positive newborn neurons in the poststroke pia mater and cortex from the early poststroke period. These findings were consistent with a previous report that demonstrated cortical neurogenesis from the acute phases after ischemia *in vivo* [12]. Although the cells isolated from the poststroke pia mater could differentiate into mature neurons expressing microtubule-associated protein 2 (MAP2) *in vitro* [2], the DCX-positive immature neurons developing in the poststroke pia mater and cortex decreased at later time points (Fig. 2H) and no MAP2-positive mature neurons were present in these regions until 60 days after stroke *in vivo* (data not shown). These findings were consistent with our previous study, which showed that mature neurons from iNSPCs could be observed only in the peristroke cortex [9,10] and not in the poststroke cortex [1]. Overall, these results may suggest that local factors present in the *in vivo* poststroke milieu [13,14] prevent newborn neurons from surviving for a long period; hence, they cannot differentiate into mature neurons. It would be challenging to accomplish cortical neurogenesis within the poststroke cortex after permanent ischemia *in vivo*; however, an abundance of DCX-positive immature neurons could develop in the poststroke pia mater and cortex if poststroke brains were incubated in a medium containing growth factors. These results suggest that cortical neurogenesis within the poststroke area may become a reality in future via the control of multiple factors.

Although the precise source, lineage, and traits of leptomeningeal progenitors, including DCX- and nestin-positive

FIG. 2. DCX-positive cells were observed near nestin-positive cells in the poststroke pia mater and cortex (DCX: **A**, red; nestin: **A**, green; DAPI: **A**, blue), although they were not observed in the nonischemic ipsilateral pia mater and cortex (DCX: **B**, red; nestin: **B**, green; DAPI: **B**, blue). Some pial cells, which were labeled with a GFP-expressing vector (**C**, GFP: **D**, **E**, green; laminin: **E**, red; DAPI: **D**, **E**, blue), migrated into the poststroke cortex after ischemia where they expressed nestin (arrows) (GFP: **F**, green; nestin: **F**, red; DAPI: **F**, blue) and DCX (arrows) (GFP: **G**, green; DCX: **G**, red; DAPI: **G**, blue). However, the number of DCX-positive cells in the poststroke pia mater and cortex decreased gradually at later time points (**H**). To investigate the potential contribution of leptomeningeal cells to cortical repair, brain slices were incubated in a medium that accelerated the proliferation of pial iNSPCs. Although DCX immunohistochemistry using the DAB reaction showed that DCX-positive cells were not observed in the nonischemic ipsilateral pia mater and cortex (**I**), it was confirmed that DCX-positive cells could survive and proliferate in the poststroke pia mater and cortex (**J**) as well as in the expanding nestin-positive cells (nestin: **K**, **M**, green; DCX: **L**, **M**, red; DAPI: **K**–**M**, blue). Scale bar: 100 μ m (**D**, **I**, and **K**) and 50 μ m (**A**, **E**, and **F**). Results shown are representative of 5 replicates of the experimental protocol. DAB, diaminobenzidine; GFP, green fluorescent protein; iNSPCs, ischemia-induced neural stem/progenitor cells.

cells, remain unclear, we showed that pial iNSPCs may originate partially from the neural crest–pericyte lineage [2], suggesting that they are indeed a novel NSPC population. Experimental lineage labeling of the leptomeninges, pericytes, and/or neural crests by genetic means may help clarify the precise characteristics of leptomeningeal cells in the poststroke brain in future studies. Accumulating evidence in the field of cardiology has also shown that cardiac stem/progenitor cells residing in the epicardium, known as epicardial progenitor cells, are activated in the adult heart after injury and that they give rise to de novo cardiomyocytes [15,16]. These findings may indicate that stem/progenitor cells are present on the surfaces of multiple organs, in addition to those observed in the CNS [2,8,17] and heart [15,16]. There are additional issues and questions to be addressed. However, the results of recent studies on leptomeninges-associated NSPCs [2,8,17] and the present study support the hypothesis that the leptomeninges that cover the entire CNS, including the brain [2] and spinal cord [8], may give rise to immature neuronal cells with an important role in CNS

restoration. Thus, the leptomeninges may become a new target for treatment of various CNS diseases, including brain and spinal cord injuries.

Materials and Methods

Induction of focal cerebral ischemia

All procedures were approved by the Animal Care Committee of Hyogo College of Medicine. Six-week-old male CB-17/ICr-+/+Jcl mice (Clea Japan, Inc.) were subjected to cerebral ischemia. Permanent focal cerebral ischemia was induced by ligation of the distal portion of the left MCA [1,2,9,10,13,18]. In brief, the left MCA was isolated, electrocauterized, and disconnected immediately distal to the point where it crossed the olfactory tract (distal M1 portion), under halothane inhalation. The infarct area in mice with this background is known to be highly reproducible and limited to the ipsilateral cerebral cortex [1,2,9,10,13,18]. Quantitative analyses were performed by investigators who were blinded to the experimental protocol and the identities of the study samples.

Histological analysis

Immunohistochemistry was performed as described previously [1,2,9,10]. In brief, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg) and perfused transcardially with 4% paraformaldehyde. Coronal brain sections (20 μ m) were prepared and stained with antibodies to nestin (Chemicon), vimentin (Santa Cruz Biotechnology), laminin (Sigma), MAP2 (Chemicon), and DCX (LifeSpan Biosciences). Primary antibodies were visualized using Alexa Fluor 488- or 555-conjugated secondary antibodies (Molecular probes). Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI) (Kirkegaard & Perry Laboratories). Images of sections were captured using a confocal laser microscope (LSM510; Carl Zeiss). The poststroke pia mater or cortex was measured (total of 50 data points/group, ie, 10 sections/mouse, $n=5$) using Image J software downloaded from NIH Image, and the number of DCX-positive cells was counted as described previously [1,2,10,13]. Results were reported as the mean $\pm$ standard deviation. To trace the leptomeningeal cells after ischemia, the mice were anesthetized and placed in a stereotactic apparatus, before a lentiviral vector encoding GFP [5.2×10^5 transducing unit (TU)/ μ L] [19] was injected into the pia mater of the MCA area (0.3 μ L, at 2.5-mm lateral and 1.5-mm rostral from the bregma) as described previously [1,2,9]. To evaluate the efficiency of GFP transduction in leptomeninges, coronal sections (20 μ m) located $\pm 200 \mu$ m anterior–posterior from the injection site were prepared and the ratio of GFP-positive cells to leptomeningeal cells was quantified in the poststroke pia mater. In another set of experiments, whole brains were removed after ischemia and coronal brain slices (6-mm thick) were incubated in Dulbecco's modified Eagle's medium (Invitrogen) containing 20 ng/mL of bFGF (Peprotech) and 20 ng/mL of EGF (Peprotech). Brain sections were then fixed, cut into 20- μ m-thick sections with a cryostat, and subjected to immunohistochemistry [2].

Reverse transcription–polymerase chain reaction

Total RNA was extracted from the poststroke pia mater, and cDNA was amplified under the following conditions [2]: 15 s at 94°C, 30 s at 56°C, and 1 min at 68°C (40 cycles). The primer sequences were as follows: DCX forward: AGAGGG TCACGGATGAATGGA and DCX reverse: GTGGGCACTA TGAGTGGGAC (amplicon size, 48 bp).

Western blot analysis

Pial cells were isolated from the poststroke leptomeninges at 3 days after stroke. Next, they were incubated in medium containing bFGF and EGF in an adherent monolayer culture [2,9], followed by incubation under differentiating conditions [2,10]. The cells were collected after trypsin treatment and samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Separated proteins (10 μ g) were transferred electrophoretically onto nitrocellulose membranes. The membranes were incubated with anti-DCX antibody (LifeSpan Biosciences) and peroxidase-labeled secondary antibodies. Antibody labeling of protein bands was detected using enhanced chemiluminescence reagents (Chemi-Lumi One; Nacalai Tesque) according to the manufacturer's instructions.

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Author Disclosure Statement

The authors have declared that no conflict of interest exists.

References

1. Nakagomi T, A Taguchi, Y Fujimori, O Saino, A Nakano-Doi, S Kubo, A Gotoh, T Soma, H Yoshikawa, et al. (2009). Isolation and characterization of neural stem/progenitor cells from post-stroke cerebral cortex in mice. *Eur J Neurosci* 29:1842–1852.
2. Nakagomi T, Z Molnar, A Nakano-Doi, A Taguchi, O Saino, S Kubo, M Clausen, H Yoshikawa, N Nakagomi and T Matsuyama. (2011). Ischemia-induced neural stem/progenitor cells in the pia mater following cortical infarction. *Stem Cells Dev* 20:2037–2051.
3. Doetsch F, I Caille, DA Lim, JM Garcia-Verdugo and A Alvarez-Buylla. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97: 703–716.
4. Moreno-Manzano V, FJ Rodriguez-Jimenez, M Garcia-Rosello, S Lainez, S Erceg, MT Calvo, M Ronaghi, M Lloret, R Planells-Cases, JM Sanchez-Puelles and M Stojkovic. (2009). Activated spinal cord ependymal stem cells rescue neurological function. *Stem Cells* 27:733–743.
5. Shimada IS, BM Peterson and JL Spees. (2010). Isolation of locally derived stem/progenitor cells from the peri-infarct area that do not migrate from the lateral ventricle after cortical stroke. *Stroke* 41:e552–e560.
6. Zawadzka M, LE Rivers, SP Fancy, C Zhao, R Tripathi, F Jamen, K Young, A Goncharevich, H Pohl, et al. (2010). CNS-resident glial progenitor/stem cells produce Schwann cells as well as oligodendrocytes during repair of CNS demyelination. *Cell Stem Cell* 6:578–590.
7. Kondo T and M Raff. (2000). Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. *Science* 289:1754–1757.
8. Decimo I, F Bifari, FJ Rodriguez, G Malpeli, S Dolci, V Lavarini, S Pretto, S Vazquez, M Sciancalepore, et al. (2011). Nestin- and doublecortin-positive cells reside in adult spinal cord meninges and participate in injury-induced parenchymal reaction. *Stem Cells* 29:2062–2076.
9. Nakagomi N, T Nakagomi, S Kubo, A Nakano-Doi, O Saino, M Takata, H Yoshikawa, DM Stern, T Matsuyama and A Taguchi. (2009). Endothelial cells support survival, proliferation, and neuronal differentiation of transplanted adult ischemia-induced neural stem/progenitor cells after cerebral infarction. *Stem Cells* 27:2185–2195.
10. Nakano-Doi A, T Nakagomi, M Fujikawa, N Nakagomi, S Kubo, S Lu, H Yoshikawa, T Soma, A Taguchi and T Matsuyama. (2010). Bone marrow mononuclear cells promote proliferation of endogenous neural stem cells through vascular niches after cerebral infarction. *Stem Cells* 28:1292–1302.

11. Boekhoorn K, A Sarabdjitsingh, H Kommerie, K de Punder, T Schouten, PJ Lucassen and E Vreugdenhil. (2008). Doublecortin (DCX) and doublecortin-like (DCL) are differentially expressed in the early but not late stages of murine neocortical development. *J Comp Neurol* 507:1639–1652.
12. Ohira K, T Furuta, H Hiohi, KC Nakamura, E Kuramoto, Y Tanaka, N Funatsu, K Shimizu, T Oishi, et al. (2010). Ischemia-induced neurogenesis of neocortical layer 1 progenitor cells. *Nat Neurosci* 13:173–179.
13. Saino O, A Taguchi, T Nakagomi, A Nakano-Doi, S Kashiwamura, N Doe, N Nakagomi, T Soma, H Yoshikawa, et al. (2010). Immunodeficiency reduces neural stem/progenitor cell apoptosis and enhances neurogenesis in the cerebral cortex after stroke. *J Neurosci Res* 88:2385–2397.
14. Takata M, T Nakagomi, S Kashiwamura, A Nakano-Doi, O Saino, N Nakagomi, H Okamura, O Mimura, A Taguchi and T Matsuyama. (2011). Glucocorticoid-induced TNF receptor-triggered T cells are key modulators for survival/death of neural stem/progenitor cells induced by ischemic stroke. *Cell Death Differ* [Epub ahead of print]; DOI: 10.1038/cdd.2011.145.
15. Zhou B, Q Ma, S Rajagopal, SM Wu, I Domian, J Rivera-Feliciano, D Jiang, A von Gise, S Ikeda, KR Chien and WT Pu. (2008). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature* 454: 109–113.
16. Smart N, S Bollini, KN Dube, JM Vieira, B Zhou, S Davidson, D Yellon, J Riegler, AN Price, MF Lythgoe, WT Pu and PR Riley. (2011). *De novo* cardiomyocytes from within the activated adult heart after injury. *Nature* 474:640–644.
17. Bifari F, I Decimo, C Chiamulera, E Bersan, G Malpeli, J Johansson, V Lisi, B Bonetti, G Fumagalli, G Pizzolo and M Krampera. (2009). Novel stem/progenitor cells with neuronal differentiation potential reside in the leptomeningeal niche. *J Cell Mol Med* 13:3195–3208.
18. Taguchi A, T Soma, H Tanaka, T Kanda, H Nishimura, H Yoshikawa, Y Tsukamoto, H Iso, Y Fujimori, et al. (2004). Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. *J Clin Invest* 114:330–338.
19. Kubo S, MC Seleme, HS Soifer, JL Perez, JV Moran, HH Kazazian Jr. and N Kasahara. (2006). L1 retrotransposition in nondividing and primary human somatic cells. *Proc Natl Acad Sci U S A* 103:8036–8041.

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