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TRANSCRIPTION FACTOR PROTEIN EXPRESSION PATTERNS BY NEURAL OR NEURONAL PROGENITOR CELLS OF ADULT MONKEY SUBVENTRICULAR ZONE

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Abstract—The anterior subventricular zone of the adult mammalian brain contains progenitor cells which are upregulated after cerebral ischemia. We have previously reported that while a part of the progenitors residing in adult monkey anterior subventricular zone travels to the olfactory bulb, many of these cells sustain location in the anterior subventricular zone for months after injury, exhibiting a phenotype of either neural or neuronal precursors. Here we show that ischemia increased the numbers of anterior subventricular zone progenitor cells expressing developmentally regulated transcription factors including Pax6 (paired-box 6), Emx2 (empty spiracles-homeobox 2), Sox 1–3 (sex determining region Y-box 1–3), Ngn1 (neurogenin 1), Dlx1,5 (distalless-homeobox 1,5), Olig1,3 (oligodendrocyte lineage gene 1,3) and Nkx2.2 (Nk-box 2.2), as compared with control brains. Analysis of transcription factor protein expression by sustained neural or neuronal precursors in anterior subventricular zone revealed that these two cell types were positive for characteristic sets of transcription factors. The proteins Pax6, Emx2, Sox2,3 and Olig1 were predominantly localized to dividing neural precursors while the factors Sox1, Ngn1, Dlx1,5, Olig2 and Nkx2.2 were mainly expressed by neuronal precursors. Further, differences between monkeys and non-primate mammals emerged, related to expression patterns of Pax6, Olig2 and Dlx2. Our results suggest that a complex network of developmental signals might be involved in the specification of primate progenitor cells. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: cerebral ischemia, primate, adult neurogenesis, cell fate, developmental signal.

The subventricular zone of the anterior horn of the lateral ventricle (SVZa) in the brain of adult mammals contains multipotent neural progenitor cells which are a subject of intensive research, predominantly using rodent models (reviewed by Gage, 2000; Okano 2002). Although studied less extensively in adult primates, SVZa precursor cells were documented also at the primate level, *in vitro* (Pincus et al., 1998; Roy et al., 2000) and *in vivo* (Kornack and Rakic, 2001; Pencea et al., 2001). Cerebral ischemia increases the proliferation of the precursor cells residing in SVZa, in both focal (Jin et al., 2001; Zhang et al., 2001; Arvidsson et al., 2002; Parent et al., 2002) and global (Iwai et al., 2003) rodent models. Recently, we confirmed the preservation of this phenomenon in primates by labeling precursor cells in adult macaque monkey brains with the thymidine analog 5-bromo-2′-deoxyuridine (BrdU). We found that the proliferation of monkey SVZa progenitors peaked early after ischemia, and that while most of these cells were destined for the olfactory bulb, some precursors retained an immature phenotype and location in SVZa for months after injury (Tonchev et al., 2005). A similar phenomenon of sustained progenitor existence in SVZa had been previously described in developing monkey brain (Ourednik et al., 2001), but the molecular signals involved in this precursor cell retention as well as in the overall regulation of primate SVZa progenitors remain unknown.

A central role in the regulation of neural development is played by families of region- and cell type-selective transcription factors that determine fundamental decisions regarding the behavior and fate of selective progenitor cell populations in the embryonic brain (reviewed by Monuki and Walsh, 2001; Shirasaki and Pfaff, 2002; Schuurmans and Guillemot, 2002). Expression of several of these developmental signals has been shown in adult rodent SVZa precursors, including paired-box (Pax) 6 (Heins et al., 2002; Hack et al., 2004), empty spiracles-homeobox (Emx) 2 (Galli et al., 2002), distalless-homeobox (Dlx) 2 (Doetsch et al., 2002), sex determining region Y-box (Sox) 2 (Ferri et al., 2004; Komitova and Eriksson, 2004), and oligodendrocyte lineage gene (Olig) 2 (Hack et al., 2004). At present, however, little is known of transcription factor expression by progenitor cells in adult primate SVZa.

In the present study, we investigated whether BrdU-positive ($BrdU^+$) progenitor cells in adult monkey SVZa express developmentally-regulated transcription factors, under normal conditions or after ischemia. We combined BrdU labeling at both short- and long-term intervals after ischemia with immunostaining for Ki67 to identify progenitors in active phases of cell cycle, or with markers of

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; Dlx, distalless-homeobox; Emx, empty spiracles-homeobox; GFAP, glial fibrillary acidic protein; Ngn, neurogenin; Nkx, Nk-box; Olig, oligodendrocyte lineage gene; Pax, paired-box; Sox, sex determining region Y-box; SVZa, anterior subventricular zone; TRITC, tetramethylrhodamine isothiocyanate.

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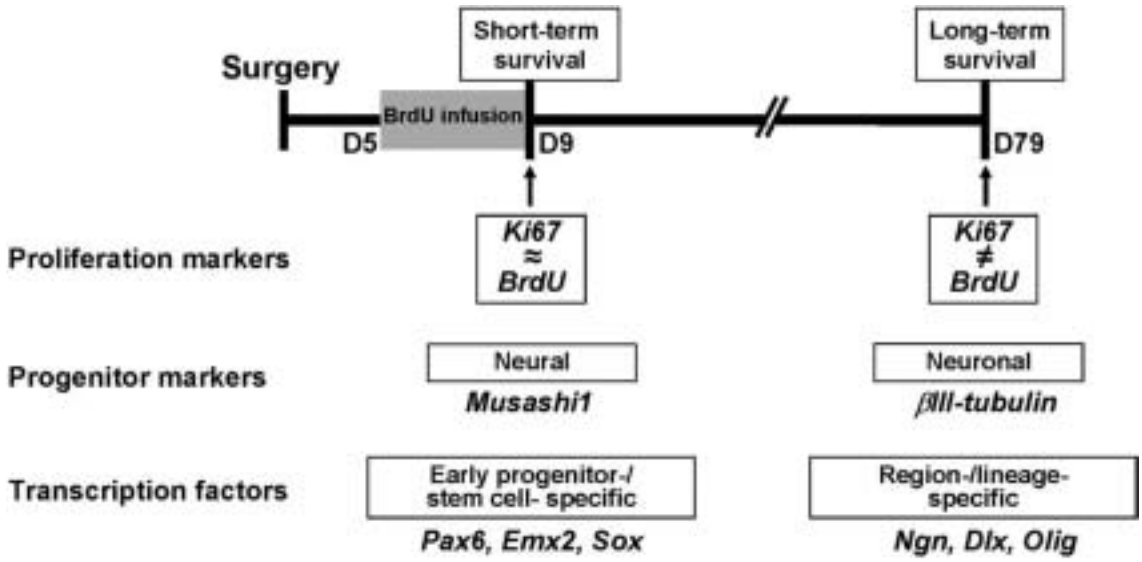

Fig. 1. Schematic presentation of BrdU/Ki67 paradigm and the investigation of transcription factor expression by progenitor cells in SVZa. BrdU infusion (gray bar) was performed between days 5–9 (D5–D9) after surgery, and the fate of $BrdU^+$ cells in SVZa was investigated on D9 (short-term interval) or at long-term intervals (D23, D44 and D79; D23 and D44 are omitted for clarity). As Ki67 selectively labels the proliferating cells at time of animal kill (arrows), at the D9 time-point almost all $BrdU^+$ cells are also $Ki67^+$, while at the long-term intervals after surgery only few $BrdU^+$ are co-labeled by Ki67. Cellular phenotypes characterized by their positivity for BrdU/Ki67 and Musashi1/βIII-tubulin (neural/neuronal) were investigated for putative expression of either early- or lineage-selective transcriptional regulators of embryonic brain development.

neural (Musashi1; Sakakibara et al., 1996; Sakakibara and Okano, 1997; Kaneko et al., 2000) or neuronal (βIII-tubulin; Pencea et al., 2001) progenitors to discern which is the cell type expressing a certain transcription factor (Fig. 1). Transcription factors targeted in our study were selected based on their expression by precursor cells in embryonic vertebrate telencephalon. We first explored markers of early multipotential stem/progenitor cells: Pax6 (Muzio et al., 2002), Emx2 (Heins et al., 2001), and Sox1–3 (Bylund et al., 2003). We then investigated transcription factors which are known to be specific for region- or lineage-restricted forebrain precursors: dorsal telencephalic markers such as neurogenin (Ngn) proteins (Schuurmans and Guillemot, 2002; Schuurmans et al., 2004), and ventral telencephalic markers such as Dlx (Anderson et al., 1997; Letinic et al., 2002; Stenman et al., 2003; Perera et al., 2004) and Olig (Schuurmans and Guillemot, 2002; Rowitch, 2004) proteins (Fig. 1). Our results suggest that some of these transcriptional regulators are expressed by adult primate SVZa progenitor cells and thus may be involved in their regulation.

EXPERIMENTAL PROCEDURES

Monkeys

Animal experiments were performed under the guidelines of the Animal Care and Ethics Committee of Kanazawa University, and the NIH Guide for the Care and Use of Laboratory Animals. Throughout the experiments, all efforts were made to minimize the number of animals used, and their suffering. Sexually mature female Japanese monkeys (*Macaca fuscata*) ($n=14$) were bred in air-conditioned cages, and were allowed free daily access to food and water. Transient global cerebral ischemia was performed under general inhalation anesthesia with artificial ventilation as previously described (Yamashima, 2000; Yamashima et al., 1998; Tonchev et al., 2005). Briefly, after resecting the sternum, the innominate and left subclavian arteries were transiently clipped for 20 min. The effectiveness of clipping was demonstrated by an almost complete absence (0.5±1.0 ml/100 g brain/min) of cerebral blood flow being monitored by laser Doppler (Vasamedics, St. Paul, MN, USA). Ischemia was performed to eight macaques, while six macaques underwent sham surgery (executed by opening the chest without vessel clipping). All monkeys received five daily injections of 100 mg/kg i.v. of BrdU (Sigma-Aldrich Japan K.K., Tokyo, Japan), performed on days 5–9 after surgery. Respective animals were then killed on day 9 ($n=2$), day 23 ($n=2$), day 44 ($n=2$) and day 79 ($n=2$) after ischemia or on day 9 ($n=2$), day 23 ($n=2$) and day 44 ($n=2$) after the sham operation (Tonchev et al., 2005).

Tissue processing

The monkeys were killed by intracardial perfusion with 4% paraformaldehyde under general anesthesia. The brains were removed, and tissue blocks (ac +7 mm anteriorly to ac +1 mm posteriorly) were cryoprotected in sucrose, and frozen in O.C.T. medium (Tissue-Tek, Sakura Finetech Co, Tokyo, Japan), and serially cut into 40-μm thick coronal sections. All stainings were performed on free-floating sections. To reveal BrdU incorporated into the cells, DNA was denatured by treatment with formamide and HCl as described (Eriksson et al., 1998; Tonchev et al., 2003), followed by application of mouse anti-BrdU (1:100, Becton Dickinson, San Jose, CA, USA) or rat anti-BrdU (1:100, Harlan Sera-Laboratory, Loughborough, UK) antibodies. We used the following antibodies for phenotypic markers: mouse anti-Ki67 (1:50, Novocastra, Newcastle, UK), rat anti-Musashi1 (1:100, Kaneko et al., 2000), rabbit anti-Musashi1 (1:200, Chemicon, Temecula, CA, USA), rabbit or mouse anti-Nestin (1:200, Chemicon), mouse anti-NeuN (1:100, Chemicon), rabbit or mouse anti-β-tubulin class III (1:400, Covance, Richmond, CA, USA), goat anti-Doublecortin (1:200; Santa Cruz Inc., Santa Cruz, CA, USA), mouse anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (1:400; Chemicon),