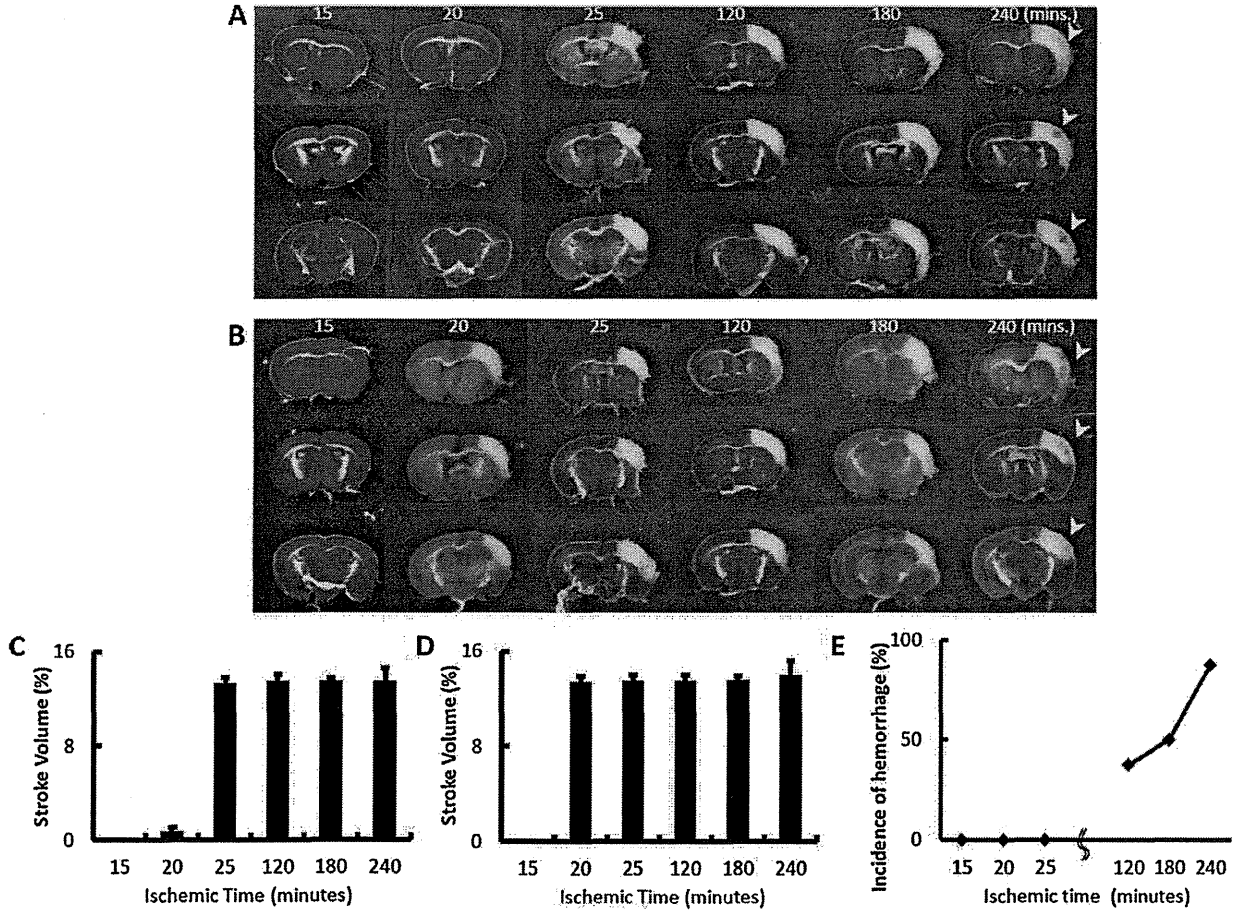

Fig. 2. Direct, transient MCA occlusion induces highly reproducible and selective cortical infarction. (A, B) Representative images of TTC-stained brain slices at 24 h (A) and 48 h (B) after ischemia/reperfusion. Though no TTC-negative area was observed after 15 min ischemia, reproducible infarction was observed after 25, 120, 180 and 240 min ischemia. It is notable that expansion of the TTC-negative area was observed at 48 h, compared with 24 h after ischemia, in mice subjected to a 20 min ischemic period. (C, D) Stroke volume at 24 h (C) and 48 h (D) after induction of ischemia. (E) Incidence of hemorrhage at 24 h after ischemia. Scale bar, 2 mm (A).

1989), the latter used as a standard for focal ischemia/reperfusion (Gomi et al., 2012; Matsuda et al., 2011; Narantuya et al., 2010). However, the intraluminal model has major disadvantages in terms of reproducibility of the area/degree of ischemia, as well as variable longer-term survival. Because the thread is inserted through left ICA, it is inevitable that blood flow is reduced to the left PCA. Furthermore, the degree flow reduction varies significantly between animals depending on the shape/placement of the thread and size of the communicating artery between the ICA and the PCA (Chiang et al., 2011; Engel et al., 2011; Kitagawa et al., 1998; Kuge et al., 1995; Longa et al., 1989; Memezawa et al., 1992). Neuronal damage may extend to the cerebral cortex and overlying the striatum depending on the time/degree of ischemia (Kanemitsu et al., 2002; Li et al., 1992; Memezawa et al., 1992). Longer periods of ischemia often cause neuronal death outside of the MCA territory, including the amygdala, hypothalamus and thalamus (Kanemitsu et al., 2002) with significant variation between animals. More extensive brain damage caused by longer ischemic periods are associated with decreased survival; the survival rate is reported to be about 10% after 240 min ischemia, respectively (Bannister and Chapman, 1984).

In contrast, direct temporal occlusion of MCA in CB-17 mice by twisting artery with thin monofilament has significant advantages.

(A) Because there is little inter-animal variation in the anatomy of the cerebral vasculature in CB-17 mice and twisting the artery with a thin monofilament completely blocks blood flow, the area and degree of ischemia are identical between animals. Such reproducibility bodes well for valid comparisons of different experimental treatments using smaller numbers of mice.

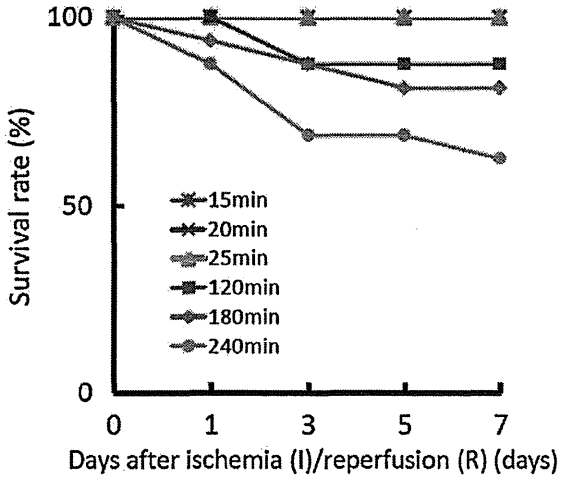

Fig. 3. Survival rate after transient ischemia. Survival rate up to 7 days after induction of transient ischemia. It is notable that the survival rate after 240 min of transient ischemia was more than 60%, even with 90% of the animals displaying hemorrhagic infarction.

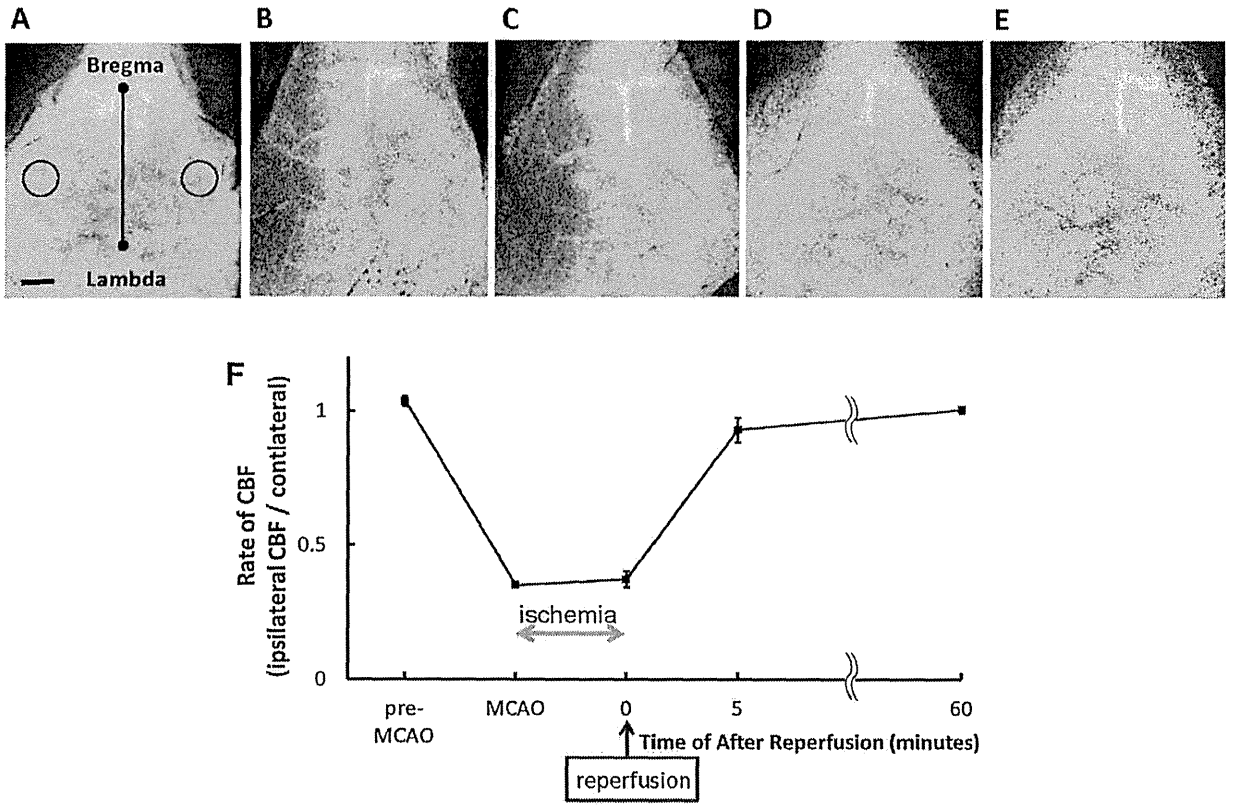

Fig. 4. Temporal changes in cerebral blood flow. (A–E) Representative pictures of two-dimensional laser speckle images before MCA occlusion (pre-MCAO: A), soon after MCA occlusion (MCAO: B), just before reperfusion (0 min: C), 5 min (D) and 60 min (E) after reperfusion. (F) The temporal changes of cerebral blood flow at ischemic core were quantified by laser speckle image. Scale bar, 1 mm (A).

(B) Because ischemic brain damage is limited to the cerebral cortex of the MCA area, survival rate at day 7 was more than 60%, even in mice subjected to 240 min of ischemia. Thus, assessment of longer-term outcomes of experimental treatments is possible.
(C) Because reproducible transient ischemia can occur for up to 240 min, a range of pathologic conditions can be modeled, including reversible reperfusion injury, delayed neuronal death, necrotic brain injury and hemorrhagic infarction.
(D) Because CB-17 mice are easy to procure, require no special diet or pre-treatment regimen and the surgical procedure is not technically demanding (compared with other methods), the current method is accessible to a wide range of investigators.

There are also short-comings of our model. The most obvious is that craniotomy results in a severe global stress to the animals, and trauma to local tissues also occurs. Sometimes, there can be small cerebral infarcts accompanying craniotomy, though these do not produce consistent functional deficits in the direct MCA occlusion model (Chen et al., 1986; Roof et al., 2001).

In conclusion, our ischemia/reperfusion model induced by twisting the MCA using a monofilament in CB-17 mice has significant advantages in reproducibility of the degree of ischemia and the ischemic area, as well as longer-term survival of the animals. Thus, it is possible to more easily test potential future therapeutic methods applicable to patients with a range of cerebral injuries using the method described herein.

Disclosures

None.

Acknowledgement

This work was supported by Japan Cardiovascular Research Foundation.

References

Bannister, C.M., Chapman, S.A., 1984. Ischemia and revascularization of the middle cerebral territory of the rat brain by manipulation of the blood vessels in the neck. Surg. Neurol. 21, 351–357.
Chan, P.H., 1996. Role of oxidants in ischemic brain damage. Stroke 27, 1124–1129.
Chen, S.T., Hsu, C.Y., Hogan, E.L., Maricq, H., Balentine, J.D., 1986. A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction. Stroke 17, 738–743.
Chiang, T., Messing, R.O., Chou, W.H., 2011. Mouse model of middle cerebral artery occlusion. J. Vis. Exp. 48, 2761.
del Zoppo, G.J., Mabuchi, T., 2003. Cerebral microvessel responses to focal ischemia. J. Cereb. Blood Flow Metab. 23, 879–894.
del Zoppo, G.J., Schmid-Schonbein, G.W., Mori, E., Copeland, B.R., Chang, C.M., 1991. Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. Stroke 22, 1276–1283.
Engel, O., Kolodziej, S., Dirnagl, U., Prinz, V., 2011. Modeling stroke in mice – middle cerebral artery occlusion with the filament model. J. Vis. Exp. 47, 2423.
Gomi, M., Takagi, Y., Morizane, A., Doi, D., Nishimura, M., Miyamoto, S., Takahashi, J., 2012. Functional recovery of the murine brain ischemia model using human induced pluripotent stem cell-derived telencephalic progenitors. Brain Res. 1459, 52–60.
Kanemitsu, H., Nakagomi, T., Tamura, A., Tsuchiya, T., Kono, G., Sano, K., 2002. Differences in the extent of primary ischemic damage between middle cerebral artery coagulation and intraluminal occlusion models. J. Cereb. Blood Flow Metab. 22, 1196–1204.
Kasahara, Y., Nakagomi, T., Matsuyama, T., Stern, D., Taguchi, A., 2012. Cilostazol reduces the risk of hemorrhagic infarction after administration of tissue-type plasminogen activator in a murine stroke model. Stroke 43, 499–506.
Kitagawa, K., Matsumoto, M., Yang, G., Mabuchi, T., Yagita, Y., Hori, M., Yanagihara, T., 1998. Cerebral ischemia after bilateral carotid artery occlusion and intraluminal suture occlusion in mice: evaluation of the patency of the posterior communicating artery. J. Cereb. Blood Flow Metab. 18, 570–579.

Kuge, Y., Minematsu, K., Yamaguchi, T., Miyake, Y., 1995. Nylon monofilament for intraluminal middle cerebral artery occlusion in rats. Stroke 26, 1655–1657.
Li, Y., Chopp, M., Garcia, J.H., Yoshida, Y., Zhang, Z.G., Levine, S.R., 1992. Distribution of the 72-kd heat-shock protein as a function of transient focal cerebral ischemia in rats. Stroke 23, 1292–1298.
Longa, E.Z., Weinstein, P.R., Carlson, S., Cummins, R., 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20, 84–91.
Matsuda, F., Sakakima, H., Yoshida, Y., 2011. The effects of early exercise on brain damage and recovery after focal cerebral infarction in rats. Acta Physiol. 201, 275–287.
Matsui, T., Nagafuji, T., Mori, T., Asano, T., 1997. N omega-nitro-L-arginine attenuates early ischemic neuronal damage of prolonged focal cerebral ischemia and recirculation in rats. Neurol. Res. 19, 192–203.
Memezawa, H., Smith, M.L., Siesjo, B.K., 1992. Penumbral tissues salvaged by reperfusion following middle cerebral artery occlusion in rats. Stroke 23, 552–559.
Nakano-Doi, A., Nakagomi, T., Fujikawa, M., Nakagomi, N., Kubo, S., Lu, S., Yoshikawa, H., Soma, T., Taguchi, A., Matsuyama, T., 2010. Bone marrow mononuclear cells promote proliferation of endogenous neural stem cells through vascular niches after cerebral infarction. Stem Cells 28, 1292–1302.
Narantuya, D., Nagai, A., Sheikh, A.M., Masuda, J., Kobayashi, S., Yamaguchi, S., Kim, S.U., 2010. Human microglia transplanted in rat focal ischemia brain induce neuroprotection and behavioral improvement. PLoS ONE 5, e11746.
Popp, A., Jaenisch, N., Witte, O.W., Frahm, C., 2009. Identification of ischemic regions in a rat model of stroke. PLoS ONE 4, e4764.
Roof, R.L., Schielke, G.P., Ren, X., Hall, E.D., 2001. A comparison of long-term functional outcome after 2 middle cerebral artery occlusion models in rats. Stroke 32, 2648–2657.
Shigeno, T., Teasdale, G.M., McCulloch, J., Graham, D.I., 1985. Recirculation model following MCA occlusion in rats. Cerebral blood flow, cerebrovascular permeability, and brain edema. J. Neurosurg. 63, 272–277.
Taguchi, A., Kasahara, Y., Nakagomi, T., Stern, D.M., Fukunaga, M., Ishikawa, M., Matsuyama, T., 2010. A reproducible and simple model of permanent cerebral ischemia in CB-17 and SCID mice. J. Exp. Stroke Transl. Med. 3, 28–33.
Taguchi, A., Soma, T., Tanaka, H., Kanda, T., Nishimura, H., Yoshikawa, H., Tsukamoto, Y., Iso, H., Fujimori, Y., Stern, D.M., Naritomi, H., Matsuyama, T., 2004. Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J. Clin. Invest. 114, 330–338.
Tamura, A., Graham, D.I., McCulloch, J., Teasdale, G.M., 1981. Focal cerebral ischaemia in the rat. 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. J. Cereb. Blood Flow Metab. 1, 53–60.
Zhang, R.L., Chopp, M., Chen, H., Garcia, J.H., 1994. Temporal profile of ischemic tissue damage, neutrophil response, and vascular plugging following permanent and transient (2H) middle cerebral artery occlusion in the rat. J. Neurol. Sci. 125, 3–10.

Naritomi H, Krieger DW (eds): Clinical Recovery from CNS Damage.
Front Neurol Neurosci. Basel, Karger, 2013, vol 32, pp 69–75 (DOI: 10.1159/000346416)

Experimental Evidence and Early Translational Steps Using Bone Marrow Derived Stem Cells after Human Stroke

Yukiko Kasahara · Masafumi Ihara · Akihiko Taguchi

Department of Regenerative Medicine Research, Institute of Biomedical Research and Innovation, Kobe, Japan

Abstract

Neurogenesis is principally restricted to the subventricular zone of the lateral ventricle wall and the subgranular zone of the hippocampal dentate gyrus in physiological situations. However, neuronal stem cells are known to be mobilized into the post- and peristroke area and we have demonstrated that appropriate support of these stem cells, achieved by therapeutic angiogenesis, enhances neuroregeneration followed by neuronal functional recovery in an experimental stroke model. We also found that neural stem cells are mobilized in patients after stroke, as well as in animal models. Based on these observations, we have started cell-based therapy using autologous bone marrow-derived stem/progenitor cells in patients after stroke. This review summarizes the findings of recent experimental and clinical studies that have focused on neurogenesis in the injured brain after cerebral infarction. We also refer to the challenges for future cell-based therapy, including regeneration of the aged brain.

Stroke is the third leading cause of death in developed countries after heart disease and cancer [1], and the leading cause of disability worldwide. More than 50% of stroke survivors are unable to completely recover and 20% of stroke patients require assistance with their daily activities [2]. Although thrombolysis can improve the functional outcomes of stroke patients, patients must be treated within 3 h (or 4.5) of the onset of a stroke [3] and no definitive treatment exists after that period other than rehabilitation. To improve functional recovery after stroke, clinical trials of various drugs have been conducted but have achieved either only mild or nonsignificant therapeutic effects, or have sometimes even had serious adverse effects [4, 5]. Thus, development of novel and safe therapies is eagerly awaited.

Recently, a number of studies have focused on cell-based therapies to promote the neuronal regeneration in the ischemic brain [6–8]. In this chapter, we present current

basic and clinical findings that focus on therapeutic neurogenesis after stroke. We also refer to a novel cell-based therapy that may enable regeneration of the aged brain.

Neuronal Regeneration Is Activated after Cerebral Ischemia

Neuronal tissue in the central nervous system is well known for its limited reparative/regenerative capacity. Physiologically speaking, neurogenesis is principally restricted to the subventricular zone of the lateral ventricle wall and the subgranular zone of the hippocampal dentate gyrus, where unique niche architectures permit continuous neurogenesis [9, 10]. In pathological situations, recent studies using experimental models have revealed that endogenous neurogenesis is activated around injured areas where neurogenesis does not occur under normal conditions [11]. Consistent with these findings, histopathological studies in stroke patients have pointed out the presence of neural stem/progenitor cells in the post-stroke human cerebral cortex, and that the peak in endogenous neurogenesis occurs approximately 1–2 weeks after a stroke [12]. These findings indicate the potential for a novel therapeutic strategy using injury-induced neurogenesis for functional recovery in patients with cerebral infarction.

Angiogenesis Is Essential for the Survival of Injury-Induced Neuronal Stem Cells

The post brain-injury neurogenic response eventually yields only a very small number of mature neurons, as most of them die after the initial boosting [11]. To achieve functional recovery by endogenous neuroregeneration, appropriate support for their survival is essential and angiogenesis has been proposed as the key element in this [7]. In the adult songbird, testosterone-induced angiogenesis leads to neuronal recruitment into the higher vocal center [13]. In the adult rat, endogenous neurogenesis and neovascularization occur in proximity to one another in the cortex following focal ischemia [14]. Moreover, angiogenesis and neurogenesis have been shown to use the same molecules for their regulation; sphingosine-1-phosphate, for example, plays a critical role in neurogenesis and angiogenesis during embryonic development [15]. This accumulating evidence indicates a close relationship between the vascular system and neurogenesis in the central nervous system, and recent studies have focused on the promotion of neurogenesis in association with angiogenesis [6].

Cell-Based Therapy to Enhance Neurogenesis in Ischemic Brain

To achieve angiogenesis in ischemic tissue, an approach using bone marrow-derived mononuclear cells, a rich cell source of both hematopoietic stem cells and endothelial stem/progenitor cells, has been proposed. Local transplantation of bone marrow-

Naritomi H, Krieger DW (eds): Clinical Recovery from CNS Damage.
Front Neurol Neurosci. Basel, Karger, 2013, vol 32, pp 69–75 (DOI: 10.1159/000346416)

derived mononuclear cells in experimental models of limb ischemia significantly induces angiogenesis and releases ischemic stress in experimental models [16]. Based on these results, clinical trials were initiated, and a cure for ischemic ulcer, with significant angiogenesis in ischemic limb, has been reported [17]. The potential for transplantation of bone marrow-derived mononuclear cells to myocardial ischemia patients was also investigated and demonstrated a therapeutic effect in experimental models. Clinical trials were initiated in patients with ischemic heart disease and the therapeutic potential for improvement in regional perfusion and heart function has been reported [18].

Based on these experimental and clinical findings, we investigated the effect of intravenous transplantation of bone marrow-derived mononuclear cells [19] and hematopoietic stem cells [7] in an experimental model. As a result, we found the following three effects: (a) cell therapy enhances neovascularization at the border of the ischemic zone; (b) neovascularization is essential for the survival of injury-induced neuronal stem cells, and (c) supporting the survival of endogenous neurogenesis improves functional outcomes [19]. The positive effect of bone marrow-derived mononuclear cells was negated by administration of an anti-angiogenesis reagent [19]. It is noteworthy that survival of transplanted cells was rarely observed, despite significant activation of angiogenesis by cell therapy. These findings indicate that the differentiation of the stem cells into endothelial cells in the ischemic brain is not essential for angiogenesis after stroke and therapeutic angiogenesis could be a novel therapeutic strategy to enhance functional recovery after stroke.

To examine the effects of the mobilization of hematopoietic stem cells from bone marrow by drug administration, granulocyte colony-stimulating factor was given in an experimental stroke model and found to impair functional recovery with brain atrophy and with exaggerated inflammatory response at the border of the ischemic region [20]. This result suggested that the mobilization of bone marrow cells, including both granulocytes and hematopoietic stem cells, by granulocyte colony-stimulating factor might augment the inflammatory response consequent to ischemic tissue damage. We also investigated the effect of intravenous transplantation of bone marrow-derived mesenchymal stem cells in an experimental stroke model but found only a mild or nonsignificant effect on functional recovery (unpublished data), though mesenchymal stem cells have the potential to suppress excessive inflammation [21].

In a preclinical trial, we investigated the appropriate cell numbers and optimal therapeutic time window using a highly reproducible murine stroke model [22] and found that administration of a relatively small number of bone marrow-derived mononuclear cells had a significantly beneficial effect on the regeneration of injured brain tissue [23]. Analysis of the therapeutic time window revealed that administration of bone marrow-derived mononuclear cells at 24 h after stroke had a mild or nonsignificant effect on regeneration following ischemia, but administration of these cells between day 2 and day 14 after the ischemic event had a significantly positive effect. This result may be attributed to the time lag between the onset of stroke and the peak of neurogenesis [12].

Naritomi H, Krieger DW (eds): Clinical Recovery from CNS Damage.
Front Neurol Neurosci. Basel, Karger, 2013, vol 32, pp 69–75 (DOI: 10.1159/000346416)

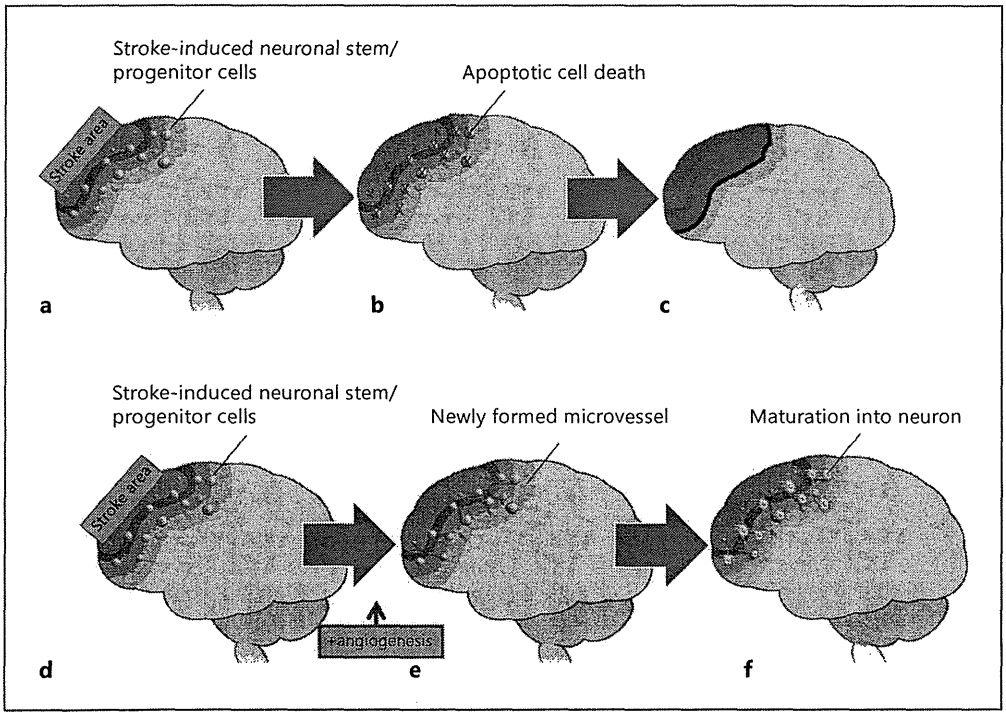

Fig. 1. Schematic representation of cell-based therapy for patients with cerebral infarction. **a–c** Neurogenesis after stroke without therapeutic angiogenesis. Endogenous neurogenesis is activated around the stroke area (**a**). However, stroke-induced neuronal stem/progenitor cells do not survive because of the lack of an appropriate environment (**b**), and do not contribute to functional recovery (**c**). **d–f** Neurogenesis with angiogenesis. Stroke-induced neuronal stem/progenitor cells (**d**) survive in an environment with angiogenesis (**e**). Neuronal stem/progenitor cells differentiate into mature neurons and contribute to functional recovery (**f**).

Clinical Trials to Enhance Neurogenesis in Patients after Stroke

Based on these results, we initiated a clinical trial to enhance neurogenesis and functional recovery through activating angiogenesis in patients with cerebral infarction. A schematic representation of this therapy is shown in figure 1. Our clinical trial is an unblinded, uncontrolled phase 1/2a study (ClinicalTrials.gov Identifier: NCT01028794). The major inclusion criteria are patients with cerebral embolism, day 7 after stroke, a National Institutes of Health Stroke Scale (NIHSS) score of more than (or equal to) 10, and an improvement in the NIHSS score of less than (or equal to) 5 since admission. On days 7–10 after stroke, either a 25-ml (low-dose group, n = 6) or a 50-ml (high-dose group, n = 6) aspiration of bone marrow cells was performed. These mononuclear cells were purified by Ficoll-Paque Premium (GE-Healthcare, USA) and administered intravenously on the day of the bone marrow aspiration. The primary outcome measures are improvement of the NIHSS score at 30 days after

Naritomi H, Krieger DW (eds): Clinical Recovery from CNS Damage.
Front Neurol Neurosci. Basel, Karger, 2013, vol 32, pp 69–75 (DOI: 10.1159/000346416)

treatment and frequency of change for the worse on the NIHSS at 30 days after treatment, compared with historical control. Though this clinical study is currently still underway, we have already treated 11 patients (6 in the low-dose and 5 in the high-dose group), and no side effects or safety problems have been observed to date. Results related to the therapeutic effects of the treatment are expected in a year. Similar clinical trials are being carried out in other countries, including the USA, UK, Brazil and Spain, with promising results [24, 25]. Though the route of administration (intravenous or intra-arterial) and cell source (bone marrow mononuclear cells or CD34-positive cells) vary, no side effects or safety problems with cell therapy have been reported. The current status of most of these ongoing clinical trials can be searched through http://clinicaltrials.gov/.

Future Cell-Based Therapy for Prevention of Cerebrovascular Diseases

Previously, we have shown that patients with cerebrovascular disease have a decreased level of circulating bone marrow-derived immature cells, the latter associated with impaired cerebrovascular function [26] and impaired cognition [27], whereas increased levels of bone marrow-derived immature cells are associated with neovascularization of the ischemic brain [28]. In addition, we have demonstrated that partial rejuvenation of bone marrow stem cells in aged rats improves vascular function and reduces ischemic damage after induction of stroke in stroke-prone spontaneously hypertensive rats [29]. Furthermore, we investigated the effect of bone marrow-derived stem cells on white matter damage in a mouse model of cerebral hypoperfusion and found that administration of bone marrow-derived stem cells has a significant protective effect against white matter damage by enhancing cerebral blood flow via the activation of nitric oxide synthase [30]. These findings clearly indicate that bone marrow-derived stem/immature cells have the potential to improve microvascular circulation and prevent cerebrovascular diseases, and the challenge to find novel strategies using autologous, allogeneic or induced pluripotent stem cell-derived hematopoietic stem cells to regenerate the aged brain is ongoing.

Conclusion

Currently, for patients after stroke, there is no specific recovery-targeted treatment other than physical and cognitive rehabilitation techniques after the period of thrombolysis. However, accumulating evidence indicates significant activation of neurogenesis after stroke, and utilization of the stroke-induced neuronal stem cells, we believe, will become a major therapeutic target for the acceleration of functional recovery. The mechanism that links angiogenesis and neurogenesis cannot be attributed to a single molecule or signaling pathway. It is likely that multiple cytokines,

Naritomi H, Krieger DW (eds): Clinical Recovery from CNS Damage.
Front Neurol Neurosci. Basel, Karger, 2013, vol 32, pp 69–75 (DOI: 10.1159/000346416)

growth factors, and cell adhesion molecules are involved, and the balance between these molecules may determine the fate of injured brain tissue. Careful, step-by-step investigation will lead to more efficient neurogenesis with a longer therapeutic time window. Experimental and clinical research focusing on neuroregeneration is needed to enhance functional recovery in patients after stroke.

References

1 Pearson TA: Cardiovascular disease in developing countries: myths, realities, and opportunities. Cardiovasc Drugs Ther 1999;13:95–104.
2 Bonita R, Solomon N, Broad JB: Prevalence of stroke and stroke-related disability. Estimates from the Auckland stroke studies. Stroke 1997;28:1898–1902.
3 Hacke W, Donnan G, Fieschi C, Kaste M, von Kummer R, Broderick JP, Brott T, Frankel M, Grotta JC, Haley EC Jr, Kwiatkowski T, Levine SR, Lewandowski C, Lu M, Lyden P, Marler JR, Patel S, Tilley BC, Albers G, Bluhmki E, Wilhelm M, Hamilton S: Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. Lancet 2004;363:768–774.
4 Adams HP Jr, Adams RJ, Brott T, del Zoppo GJ, Furlan A, Goldstein LB, Grubb RL, Higashida R, Kidwell C, Kwiatkowski TG, Marler JR, Hademenos GJ, Stroke Council of the American Stroke A: Guidelines for the early management of patients with ischemic stroke: a scientific statement from the Stroke Council of the American Stroke Association. Stroke 2003; 34:1056–1083.
5 Sandercock PA, Soane T: Corticosteroids for acute ischaemic stroke. Cochrane Database Syst Rev 2011; 9:CD000064.
6 Nakagomi N, Nakagomi T, Kubo S, Nakano-Doi A, Saino O, Takata M, Yoshikawa H, Stern DM, Matsuyama T, Taguchi A: Endothelial cells support survival, proliferation, and neuronal differentiation of transplanted adult ischemia-induced neural stem/progenitor cells after cerebral infarction. Stem Cells 2009;27:2185–2195.
7 Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, Tsukamoto Y, Iso H, Fujimori Y, Stern DM, Naritomi H, Matsuyama T: Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J Clin Invest 2004;114:330–338.
8 Takahashi K, Yasuhara T, Shingo T, Muraoka K, Kameda M, Takeuchi A, Yano A, Kurozumi K, Agari T, Miyoshi Y, Kinugasa K, Date I: Embryonic neural stem cells transplanted in middle cerebral artery occlusion model of rats demonstrated potent therapeutic effects, compared to adult neural stem cells. Brain Res 2008;1234:172–182.
9 Alvarez-Buylla A, Garcia-Verdugo JM: Neurogenesis in adult subventricular zone. J Neurosci 2002;22: 629–634.
10 Gage FH: Molecular and cellular mechanisms contributing to the regulation, proliferation and differentiation of neural stem cells in the adult dentate gyrus. Keio J Med 2010;59:79–83.
11 Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O: Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 2002;8:963–970.
12 Nakayama D, Matsuyama T, Ishibashi-Ueda H, Nakagomi T, Kasahara Y, Hirose H, Kikuchi-Taura A, Stern DM, Mori H, Taguchi A: Injury-induced neural stem/progenitor cells in post-stroke human cerebral cortex. Eur J Neurosci 2010;31:90–98.
13 Louissaint A Jr, Rao S, Leventhal C, Goldman SA: Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 2002;34: 945–960.
14 Shin HY, Kim JH, Phi JH, Park CK, Kim JE, Kim JH, Paek SH, Wang KC, Kim DG: Endogenous neurogenesis and neovascularization in the neocortex of the rat after focal cerebral ischemia. J Neurosci Res 2008;86:356–367.
15 Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL: Essential role for sphingosine kinases in neural and vascular development. Mol Cell Biol 2005;25:11113–11121.
16 Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J, Imaizumi T: Augmentation of postnatal neovascularization with autologous bone marrow transplantation. Circulation 2001;103:897–903.
17 Kirana S, Stratmann B, Prante C, Prohaska W, Koerperich H, Lammers D, Gastens MH, Quast T, Negrean M, Stirban OA, Nandrean SG, Gotting C, Minartz P, Kleesiek K, Tschoepe D: Autologous stem cell therapy in the treatment of limb ischaemia induced chronic tissue ulcers of diabetic foot patients. Int J Clin Pract 2012;66:384–393.
18 Moreira Rde C, Haddad AF, Silva SA, Souza AL, Tuche FA, Oliveira MA, Mesquita CT, Rochitte CE, Borojevic R, Dohmann HF: Intracoronary stem-cell injection after myocardial infarction: microcirculation sub-study. Arq Bras Cardiol 2011;97:420–426.

Naritomi H, Krieger DW (eds): Clinical Recovery from CNS Damage.
Front Neurol Neurosci. Basel, Karger, 2013, vol 32, pp 69–75 (DOI: 10.1159/000346416)

19 Nakano-Doi A, Nakagomi T, Fujikawa M, Nakagomi N, Kubo S, Lu S, Yoshikawa H, Soma T, Taguchi A, Matsuyama T: Bone marrow mononuclear cells promote proliferation of endogenous neural stem cells through vascular niches after cerebral infarction. Stem Cells 2010;28:1292–1302.
20 Taguchi A, Wen Z, Myojin K, Yoshihara T, Nakagomi T, Nakayama D, Tanaka H, Soma T, Stern DM, Naritomi H, Matsuyama T: Granulocyte colony-stimulating factor has a negative effect on stroke outcome in a murine model. Eur J Neurosci 2007;26:126–133.
21 Tsuda H, Yamahara K, Ishikane S, Otani K, Nakamura A, Sawai K, Ichimaru N, Sada M, Taguchi A, Hosoda H, Tsuji M, Kawachi H, Horio M, Isaka Y, Kangawa K, Takahara S, Ikeda T: Allogenic fetal membrane-derived mesenchymal stem cells contribute to renal repair in experimental glomerulonephritis. Am J Physiol Renal Physiol 2010;299:F1004–F1013.
22 Taguchi A, Kasahara Y, Nakagomi T, Stern DM, Fukunaga M, Ishikawa M, Matsuyama T: A reproducible and simple model of permanent cerebral ischemia in CB-17 and SCID mice. J Exp Stroke Transl Med 2010;3:28–33.
23 Uemura M, Kasahara Y, Nagatsuka K, Taguchi A: Cell-based therapy to promote angiogenesis in the brain following ischemic damage. Curr Vasc Pharmacol 2012;10:285–288.
24 Savitz SI, Misra V, Kasam M, Juneja H, Cox CS Jr, Alderman S, Aisiku I, Kar S, Gee A, Grotta JC: Intravenous autologous bone marrow mononuclear cells for ischemic stroke. Ann Neurol 2011;70:59–69.
25 Battistella V, de Freitas GR, da Fonseca LM, Mercante D, Gutfilen B, Goldenberg RC, Dias JV, Kasai-Brunswick TH, Wajnberg E, Rosado-de-Castro PH, Alves-Leon SV, Mendez-Otero R, Andre C: Safety of autologous bone marrow mononuclear cell transplantation in patients with nonacute ischemic stroke. Regen Med 2011;6:45–52.
26 Taguchi A, Matsuyama T, Moriwaki H, Hayashi T, Hayashida K, Nagatsuka K, Todo K, Mori K, Stern DM, Soma T, Naritomi H: Circulating CD34-positive cells provide an index of cerebrovascular function. Circulation 2004;109:2972–2975.
27 Taguchi A, Nakagomi N, Matsuyama T, Kikuchi-Taura A, Yoshikawa H, Kasahara Y, Hirose H, Moriwaki H, Nakagomi T, Soma T, Stern DM, Naritomi H: Circulating CD34-positive cells have prognostic value for neurologic function in patients with past cerebral infarction. J Cereb Blood Flow Metab 2009;29:34–38.
28 Yoshihara T, Taguchi A, Matsuyama T, Shimizu Y, Kikuchi-Taura A, Soma T, Stern DM, Yoshikawa H, Kasahara Y, Moriwaki H, Nagatsuka K, Naritomi H: Increase in circulating CD34-positive cells in patients with angiographic evidence of moyamoya-like vessels. J Cereb Blood Flow Metab 2008;28:1086–1089.
29 Taguchi A, Zhu P, Cao F, Kikuchi-Taura A, Kasahara Y, Stern DM, Soma T, Matsuyama T, Hata R: Reduced ischemic brain injury by partial rejuvenation of bone marrow cells in aged rats. J Cereb Blood Flow Metab 2011;31:855–867.
30 Fujita Y, Ihara M, Ushiki T, Hirai H, Kizaka-Kondoh S, Hiraoka M, Ito H, Takahashi R: Early protective effect of bone marrow mononuclear cells against ischemic white matter damage through augmentation of cerebral blood flow. Stroke 2010;41:2938–2943.

Akihiko Taguchi
Department of Regenerative Medicine Research
Institute of Biomedical Research and Innovation
2–2 Minatojima-Minamimachi, Chuo-ku, Kobe 650–0047 (Japan)
E-Mail taguchi@fbri.org

Naritomi H, Krieger DW (eds): Clinical Recovery from CNS Damage.
Front Neurol Neurosci. Basel, Karger, 2013, vol 32, pp 69–75 (DOI: 10.1159/000346416)

Experimental Neurology 247 (2013) 218–225

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

A novel reproducible model of neonatal stroke in mice: Comparison with a hypoxia–ischemia model

Masahiro Tsuji [a,*], Makiko Ohshima [a], Akihiko Taguchi [a,b], Yukiko Kasahara [b], Tomoaki Ikeda [c], Tomohiro Matsuyama [d]

[a] Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center Research Institute, 5-7-1, Fujishiro-dai, Suita, Osaka, 565-8565, Japan
[b] Department of Regenerative Medicine, Institute of Biomedical Research and Innovation, 2-2, Minami-machi, Minatojima, Chuo-ku, Kobe, 650-0047, Japan
[c] Department of Obstetrics and Gynecology, Mie University School of Medicine, 2-174, Edobashi, Tsu, Mie, 514-8507, Japan
[d] Laboratory of Neurogenesis and CNS Repair, Institute for Advanced Medical Science, Hyogo College of Medicine, 1-1, Mukogawacho, Nishinomiya, Hyogo, 663-8501, Japan

ARTICLE INFO

Article history:
Received 18 January 2013
Revised 1 April 2013
Accepted 18 April 2013
Available online 4 May 2013

Keywords:
Neonatal stroke
Neonatal encephalopathy
Focal ischemia
Middle cerebral artery occlusion
Hypoxic–ischemic encephalopathy
CB-17 mouse
Variability

ABSTRACT

Neonatal stroke occurs in 1/4000 live births and leaves life-long neurological impairments, such as cerebral palsy and epilepsy. Currently, the rodent models of neonatal stroke that are available exhibit significant inter-animal variability, which makes it difficult to accurately assess the mechanisms of brain injury and the efficacy of candidate treatments. We aimed to introduce a novel, highly reproducible model of stroke, middle cerebral artery occlusion (MCAO), in immature mice, and to evaluate the reproducibility of this model compared with a conventional hypoxia–ischemia (HI) model. Postnatal day 12 CB-17 mice underwent left MCAO by direct electrocoagulation. The MCAO model exhibited excellent long-term survival; 85% up to 8 weeks after the insult. Infarct was evident in every animal with MCAO ($n = 27$) and was confined to the cortex, with the exception of some mild thalamic injury. While the % stroke volume 48 h after the insult was consistent in the MCAO group, range: 17.8–30.4% (minimum–maximum), it was substantially less consistent in the HI group, range: 3.0–70.1%. This contrasting variability between the two models was also evident in the cerebral blood flow, 24 h after the insult, and in the ipsilateral hemispheric volume, as assessed at 8 weeks after the insult. Mice with MCAO exhibited significant neurofunctional deficits in the rotarod and open-field tests. Preclinical studies for neonatal stroke could become more reliable using this model, with even a potential reduction in the number of pups required for statistical significance. The contrasting variability between the two models may provide insights into the factors that contribute to inter-animal variability in brain injury.

Introduction

Perinatal/neonatal arterial ischemic stroke occurs in 1/2800 to 1/5000 live births, has a mortality rate of 2–10%, and leaves life-long neurological impairments, such as cerebral palsy, cognitive delay, and epilepsy (Chabrier et al., 2011; Golomb et al., 2006; Nelson and Lynch, 2004). The common early symptoms are seizures, persistently altered muscle tone, and decreased consciousness (Chabrier et al., 2011). Most perinatal arterial ischemic events occur in the region of the middle cerebral artery (MCA), with a left-hemisphere predominance (Lee et al., 2005; Sreenan et al., 2000). While full-term infants tend to exhibit occlusion of the main branch, preterm infants tend to exhibit occlusions of a cortical branch or one or more of the lenticulostriate branches (de Vries et al., 1997). There is currently no evidence-based treatment for neonates with stroke (Chabrier et al., 2011). Furthermore, the average 5-year direct medical cost for neonatal stroke is approximately $52,000 US (Gardner et al., 2010).

When investigating brain injuries, it is essential to utilize a highly reproducible model of brain injury. The model has to provide: 1) an accurate neurological evaluation, 2) a detailed evaluation of the injury/neuroprotection mechanisms, and 3) limitation in the numbers of animals used. Several neonatal stroke models have been developed using artery obstruction (Ashwal et al., 1995; Comi et al., 2004; Derugin et al., 1998; Mitsufuji et al., 1996; Renolleau et al., 1998; Wen et al., 2004). Almost all of these models exhibit significant inter-animal variability in the extent of the brain injury; i.e. a subset of pups exhibit no perceivable brain injury.

Neonatal encephalopathy (NE) is a neonatal neurological syndrome with clinical features that include decreased consciousness –

Abbreviations: MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; NE, neonatal encephalopathy; HIE, hypoxic–ischemic encephalopathy; HI, hypoxic–ischemic, hypoxia–ischemia; CBF, cerebral blood flow; ANOVA, analysis of variance.

* Corresponding author. Fax: +81 6 6835 5496.
E-mail addresses: tsuji.masahiro.ri@mail.ncvc.go.jp (M. Tsuji), oshima.makiko.ri@mail.ncvc.go.jp (M. Ohshima), taguchi@fbri.org (A. Taguchi), kasahara@fbri.org (Y. Kasahara), t-ikeda@clin.medic.mie-u.ac.jp (T. Ikeda), tomohiro@hyo-med.ac.jp (T. Matsuyama).

0014-4886/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.expneurol.2013.04.015

usually associated with respiratory depression, altered muscle tone, disturbances of cranial nerve function – especially impaired feeding, and often seizures (Volpe, 2012). The most common etiology of NE is cerebral ischemia; hypoxic–ischemic encephalopathy (HIE) 50–80%, and stroke ~5–10% (Volpe, 2012), NE encompasses HIE and stroke. Recently, some authors have proposed that the term HIE should not be used in practice and should be replaced by the more general term, NE, for a number of reasons (Dammann et al., 2011), whereas other authors have opposed this proposal (Volpe, 2012). The most widely-used HIE model is the Rice–Vannucci model, which combines permanent unilateral ligation of the carotid artery in 7-day-old rat pups, along with exposure to hypoxia (Johnston et al., 2005; Rice et al., 1981). It is important to note that this model also exhibits significant inter-animal variability in the extent of the brain injury (Aden et al., 2002; Sheldon et al., 1998).

Some neonates with stroke can present signs and symptoms similar to HIE, and vice-versa. Moreover, some babies may exhibit both etiologies, and it is often difficult to isolate the cause of NE. Therefore, it is important to understand the differences between arterial ischemic stroke and hypoxia–ischemia (HI). Nevertheless, to the best of our knowledge, only one study (Ashwal et al., 2007) has directly compared the HI model in immature animals and a stroke model in immature animals to date.

We have previously developed a highly reproducible model of adult stroke induced by direct electrocoagulation of the unilateral MCA in CB-17 (CB-17/Icr-+/+Jcl) and SCID (CB-17/Icr-scid/scidJcl) mice (Taguchi et al., 2004, 2010). Recently, we adapted the same technique to immature CB-17 mice, and have succeeded in developing a model of neonatal stroke that shows remarkable consistency of the brain injury. The objectives of our study were: 1) to introduce a novel model of stroke in immature mice and 2) to test reproducibility of this model as compared to the HI model.

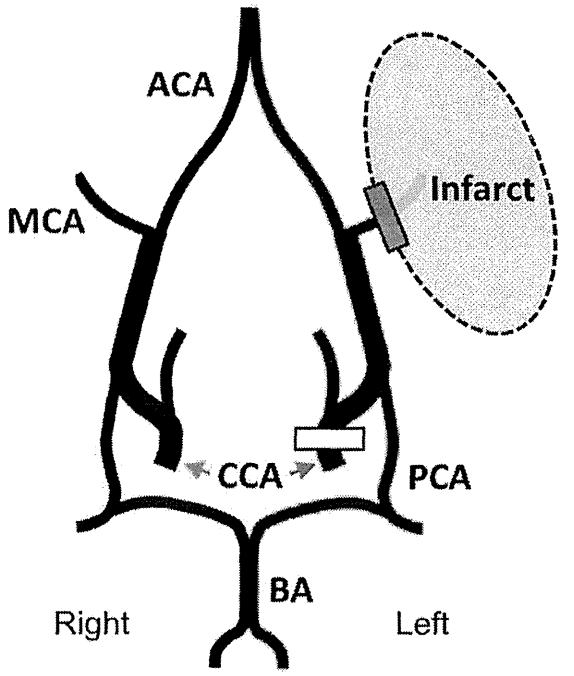

Fig. 1. Representation of the circle of Willis in rodents. The anatomic arterial system at the base of the brain in horizontal projection. ACA; anterior cerebral artery. BA; basilar artery. CCA; common carotid artery. MCA; middle cerebral artery. PCA; posterior cerebral artery. In the MCA occlusion model, the left MCA is permanently occluded (gray box). In the hypoxia–ischemia model, the left CCA is permanently occluded (open box) followed by transient systemic exposure to hypoxia.

Methods

Animals and surgeries

Postnatal day 12 (P12) male and female CB-17 mouse pups (n = 94, weight: 6.7 ± 1.2 g) (CLEA Japan Inc., Tokyo, Japan) were prepared for surgery. P8–12 mice are considered comparable to human term (P0) neonates with regard to brain maturation (Hagberg et al., 2002). All experiments were performed in accordance with protocols approved by the Experimental Animal Care and Use Committee of the National Cerebral and Cardiovascular Center.

Permanent MCA occlusion (MCAO) was produced by a modification of the adult MCAO model that we have reported previously (Taguchi et al., 2010) (Fig. 1). A skin incision was made between the left eye and ear under isoflurane anesthesia (4.0% for induction, 1.5–2.0% for maintenance). The zygoma was dissected to visualize the MCA through the cranial bone. A hole was made in the temporal bone by removing a portion of it using fine forceps. The left MCA was electrocauterized, and disconnected just distal to its crossing of the olfactory tract (distal M1 portion). The average duration of the whole procedure was approximately 15 min. HI was induced by a combination of permanent occlusion of the left common carotid artery and exposure to 8% oxygen for 30 min in the P12 CB-17 mice, as described previously (Ohshima et al., 2012) (Fig. 1). Sham-surgery controls underwent open-skull surgery without MCA electrocoagulation. To properly assess the differences in variability between the two models, a single researcher, the first author, performed all surgical procedures. All analyses were performed by investigators who were blinded to the experimental group.

Cerebral blood flow measurements

The cortical surface cerebral blood flow (CBF) was measured by a laser speckle flowmetry imaging system (Omegawave Inc., Tokyo, Japan) immediately before and 24 h after MCAO or HI, as described previously, with a minor modification (Ohshima et al., 2012). CBF was measured through the intact skull with an open-scalp.

Behavioral tests

Sensorimotor skills were evaluated 2 weeks after the insult (P26) using the rotarod test, as rodents with brain damage have been reported to exhibit behavioral impairment at this time point (Jansen and Low, 1996). The rotarod accelerated from 4 to 40 rpm over 5 min (Muromachi Kikai Co., Ltd., Tokyo, Japan). The time until the mouse fell off the rotating drum was recorded in 5 consecutive sessions, and the average time spent on the drum was used for statistical comparison.

Locomotor and exploratory behaviors were evaluated 5 weeks after the insults (almost 7 weeks of age) using the open-field test, as in our preliminary study mice began to respond to a dark environment from this age onward. Animals were allowed to search freely in a box (30 × 30 cm) for 30-min in a light environment and for the subsequent 30-min in a dark environment (Taiyo Electric Co., Ltd, Osaka, Japan). On the X-, Y-, and Z-banks of the open-field, infrared beams were mounted at specific intervals. The total number of beam crossings by the animal was counted and scored as "locomotion" for the horizontal movement, and as "rearing" for the vertical movement. Both behavioral tests were repeated one week before sacrifice at 8 weeks after the insult.

Histological analyses

Morphological evaluation of the brain injury was performed, as described previously (Tsuji et al., 2004, 2012). Forty-eight hours after the MCAO or HI insult, the brain was removed and sectioned coronally in 1-mm thick slices. The area of the viable ipsilateral and contralateral hemispheres, which stained red with 2,3,5-triphenyltetrazolium chloride (TTC) in each brain section, was measured using ImageJ software (NIH, Bethesda, USA). The hemispheric volume was estimated by integrating the hemispheric areas.

For longer-term evaluation, separate sets of animals were perfusion-fixed intracardially with 4% paraformaldehyde, 8 weeks after the insult. In assessing the hematoxylin–eosin-stained sections, neuropathological injury in the cerebral cortex was scored on a scale ranging from 0 to 4 points (0, no injury; 4, extensive confluent infarction). Neuropathologic injury in the hippocampus, striatum, and thalamus was scored on a scale ranging from 0 to 6 points. The ipsilateral and contralateral areas in the four regions and the corpus callosum were measured using ImageJ software. The ratios of the ipsilateral/contralateral areas in the five regions were calculated after summing the areas in four brain sections (cortex) or two brain sections (hippocampus, striatum, thalamus, and corpus callosum).

Statistics

The mortality rate of the animals was analyzed using Fisher's exact test with Bonferroni's correction for multiple comparisons. Hemispheric volumes, and CBF were assessed using two-way analysis of variance (ANOVA), followed by the Bonferroni test. The differences in body weight were assessed using one-way ANOVA, followed by the Bonferroni test. The injury scores were not distributed normally, so differences in injury scores were assessed with the Mann–Whitney *U* test. Ratios of the ipsilateral/contralateral areas were assessed using a Kruskal–Wallis test, followed by Dunn's multiple comparison, as the variances of the ratios were significantly different among the three groups. Pearson's product–moment correlation coefficient analysis was performed to determine the correlation between CBF and brain injury. Outcomes in the rotarod and open-field tests performed at two time points were assessed using two-way repeated measures ANOVA. Temporal changes during the course of a 60-min session in open-field test were then analyzed using two-way repeated measures ANOVA. Differences were considered significant at $P < 0.05$. The results are presented as the mean ± standard deviation (SD), unless otherwise noted.

Results

Mortality and body weight

All pups that were prepared for surgery underwent the surgery successfully. Although some pups experienced bleeding during the MCAO surgery, all pups were included in the subsequent analyses. Survival was 100% at 48 h and 85% at 8 weeks after MCAO (Table 1). Body weights at P12 and 8 weeks later did not differ among groups, including the no-surgery controls (Table 2).

Table 1
Mortality rates.

	48 h-survival cohort	8-week-survival cohort
No-surgery		0/13
Sham-surgery		2/17
HI	1/12	6/22
MCAO	0/10	3/20

None of the pups died during the surgical procedure for either MCAO (middle cerebral artery occlusion) or HI (hypoxia–ischemia). In each cohort, mortality rates did not differ significantly between groups.

Table 2
Body weights.

	Postnatal day 12	8 weeks later
No-surgery	6.5 ± 0.6	21.9 ± 2.0
Sham-surgery	6.9 ± 0.9	22.2 ± 2.1
HI	6.6 ± 1.4	20.5 ± 2.3
MCAO	6.8 ± 1.1	21.9 ± 3.2

Body weights (grams) (mean ± SD) at postnatal day 12 (the day of surgery) and 8 weeks later were not different between groups. MCAO; middle cerebral artery occlusion, HI; hypoxia–ischemia.

Morphological brain injury

Forty-eight hours after the insult, moderate-complete TTC discoloration was observed in all 10 pups that were subjected to MCAO, while discoloration was observed in only five out of 11 pups that were subjected to HI (Fig. 2A). The discoloration was confined to the ipsilateral cerebral cortex, and its location and size were consistent in all pups in the MCAO group, with the exception of one pup that exhibited discoloration extending to the striatum. In contrast, the location and size of the discoloration was markedly more variable in the HI group. The mean % stroke volume was 25.1 ± 3.6% in the MCAO group and 15.5 ± 18.6% in the HI group. The % stroke volume was calculated as follow: ((contralateral volume − viable ipsilateral volume) / contralateral volume) × 100%. Variances of the viable ipsilateral hemispheric volume and % stroke volume differed significantly between the two models ($P < 0.001$) (Fig. 2B).

Eight weeks after the insult, all 17 mice with MCAO exhibited consistent macroscopic cortical damage (Fig. 2C). The mean ipsilateral hemispheric volume was 73.0 ± 3.2 mm^3 in the MCAO group, and 72.3 ± 23.0 mm^3 in the HI group (Fig. 2D). Of note, the sham-surgery group was not different from the no-surgery group, suggesting that the open-skull surgical procedure did not cause noticeable morphological damage. No sex differences in hemispheric volumes were observed at either time point in any of the groups.

Neuropathological injury scores in the four brain regions examined differed between the two models (Fig. 3A). The ratios of the ipsilateral/contralateral areas in the four regions and corpus callosum differed among the three groups including the sham-surgery group (Fig. 3B). Interestingly, in the MCAO group, most mice exhibited mild thalamic injury, in contrast with a virtual absence of striatal or hippocampal injury. Furthermore, the thalamic damage in the MCAO model was strictly restricted to the ipsilateral ventroposterior thalamic nuclei (VPN), which contained many pyknotic cells (Fig. 3C). In contrast, the thalamic injury in the HI model was variable in terms of its distribution and severity. In both models, the ispilateral corpus callosum exhibited mild atrophy; however, this only reached statistical significance in the MCAO model.

CBF

The CBF was decreased in the MCA territory on the ipsilateral side in all pups 24 h after the HI or MCAO insult. The degree of the CBF reduction was consistent after MCAO, whereas it was variable between animals after HI (Figs. 4A, B). The CBF 24 h after the insult was compared with the morphological brain injury at 8 weeks after the insult (Fig. 4C). The reduction in CBF after the MCAO did not correlate with the subsequent morphological brain injury. In stark contrast, the reduction in CBF after the HI insult correlated strongly with brain injury ($R^2 = 0.99$), which is consistent with our previous report in P8 mice with the HI insult (Ohshima et al., 2012).

Rotarod performance

Sensorimotor performance, as assessed by rotarod treadmill at 2 and 7 weeks after the insult was analyzed by two-way repeated

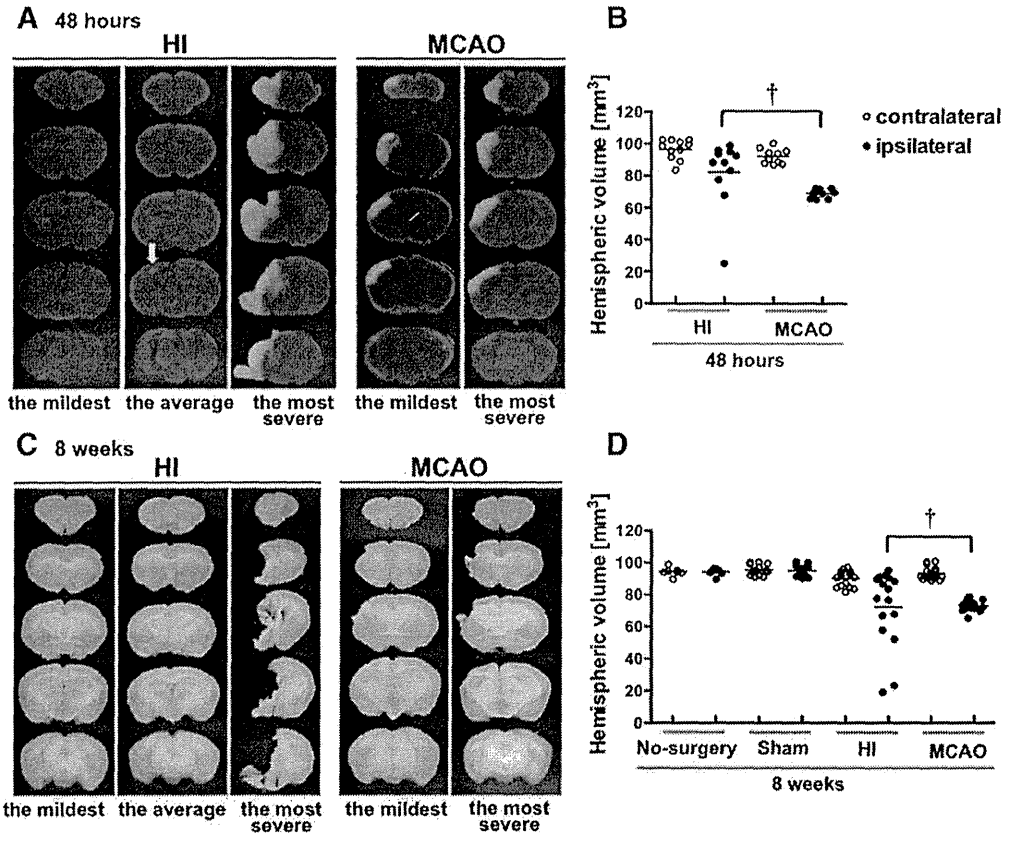

Fig. 2. Macroscopic brain injuries. (A) Images of TTC-stained brain sections 48 h after middle cerebral artery occlusion (MCAO) or hypoxia–ischemia (HI). The brains with the mildest injury and the most severe injury in the MCAO group and those with the mildest, the average, and the most severe injury in HI group are shown. The brain injury was highly consistent after MCAO. In contrast, the brain injury varied substantially after HI (the arrow indicates a small area of discoloration). (B) Hemispheric volumes of viable tissue, which stained red, examined at 48 h after the insult (HI $n = 11$; MCAO $n = 10$). (C) Images of brain slices 8 weeks after the insult. (D) Hemispheric volumes examined at 8 weeks after the insult. † Significant difference in the variances between the groups ($P < 0.001$). There were no significant differences in the ipsilateral hemispheric volumes between the no-surgery and sham-surgery groups, nor in the contralateral hemispheric volumes in the no-surgery, sham-surgery group, and MCAO groups. (no-surgery $n = 7$; sham-surgery $n = 15$; HI $n = 16$; MCAO $n = 17$).

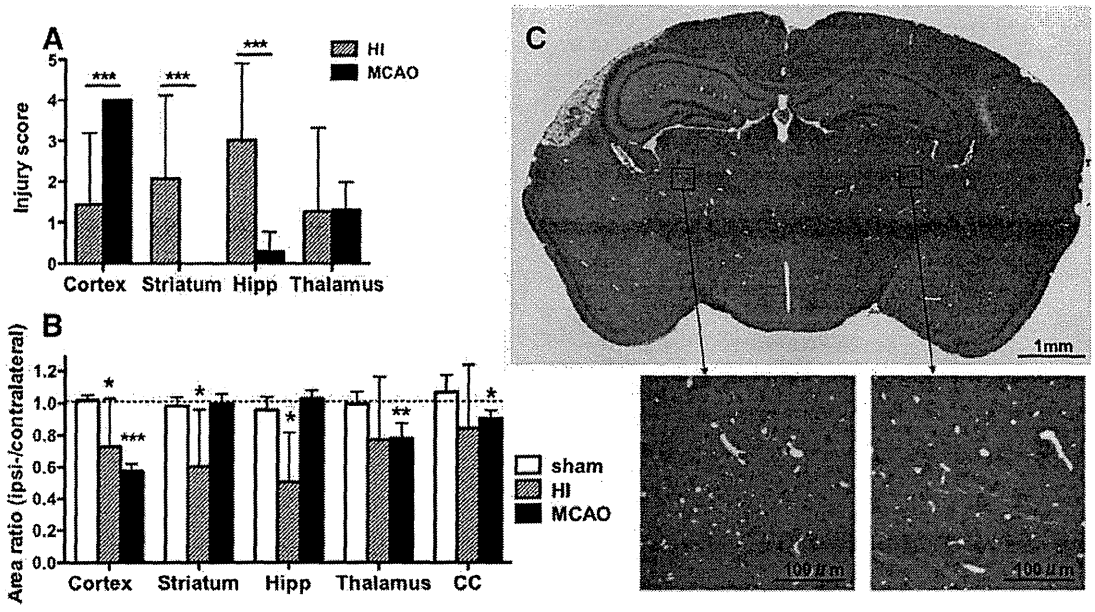

Fig. 3. Microscopic brain injuries. (A) Neuropathological injury scores examined in hematoxylin–eosin-stained sections 8 weeks after the insult. ***$P < 0.001$. (HI $n = 16$; MCAO $n = 17$) (B) The ratios of ipsilateral/contralateral areas in each region examined at 8 weeks after the insult. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with sham. Note the difference in the error bars (standard deviation) between the models (sham-surgery $n = 7$; HI $n = 10$; MCAO $n = 10$). Hipp; hippocampus. CC; corpus callosum. (C) Representative image of H&E-stained sections of mice brain 8 weeks after the MCAO. There is a clearly demarcated old infarct in the ipsilateral cortex. The ipsilateral thalamus is mildly atrophic. The labeled boxes indicate the regions that were selected for higher magnification (×20). Many pyknotic neurons are observed in the ipsilateral ventroposterior thalamic nucleus (VPN). The contralateral VPN appears normal.

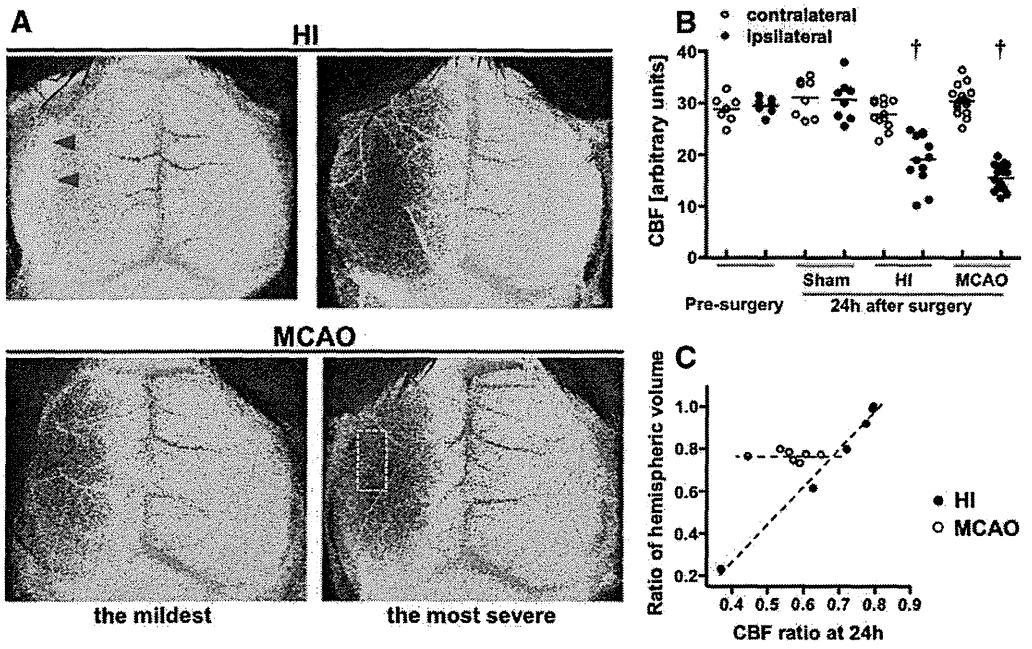

Fig. 4. Cerebral blood flow. (A) Images of the cerebral blood flow (CBF) 24 h after the insult. The reduction of the CBF, indicated by the bluish color, was consistent after MCAO, but not after HI (the arrowheads indicate the main trunk of the MCA). (B) CBF levels were measured in the ischemic core region (the box with dotted line) of the MCA territory and in the matching region on the contralateral side before and after the insult. † Significant difference compared with the pre-surgery or sham-surgery groups ($P < 0.001$), and significant difference between each model ($P < 0.01$) (pre-surgery $n = 7$; sham-surgery $n = 8$; HI $n = 12$; MCAO $n = 17$). (C) The ratio of the ipsilateral CBF to the contralateral CBF at 24 h after the insult was compared with the ratio of the ipsilateral hemispheric volume to the contralateral hemispheric volume (assessed 8 weeks after the insult). The correlation between the degree of CBF reduction and the degree of brain damage is extremely strong in the HI group ($R^2 = 0.99$). (HI $n = 6$; MCAO $n = 7$).

measure ANOVA. There were significant time and group differences; the performance in mice with MCAO was significantly impaired compared with that in the sham-surgery group (Fig. 5). The impairment in the rotarod performance in mice with HI was not statistically significant.

Open-field activities

We initially analyzed overall activities during 60-min sessions at 5 and 7 weeks after the insult using two-way repeated measures ANOVA (Figs. 6A, B). While there was no time difference with respect to either locomotion or rearing, there was a significant group difference with respect to rearing, but not locomotion; mice with HI were hypoactive compared with the mice in the other three groups.

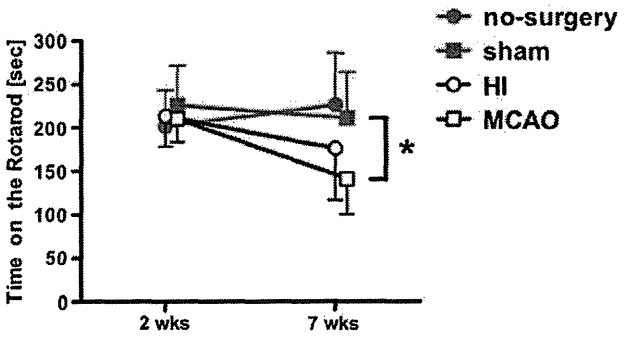

Fig. 5. Rotarod test. Repeated-measures two-way ANOVA showed significant time and group differences in sensorimotor performance, assessed 2 and 7 weeks after the insult. Performance was significantly impaired in mice with MCAO compared with the sham-surgery groups. *$P < 0.05$. (no-surgery $n = 19$; sham-surgery $n = 13$; HI $n = 16$; MCAO $n = 11$, 2 weeks after the insult. no-surgery $n = 9$; sham-surgery $n = 10$; HI $n = 13$; MCAO $n = 11$, 7 weeks after the insult).

There were no overall reductions in locomotion or rearing in the mice with MCAO.

We then analyzed the temporal changes throughout a 60-min session in 5-min increments using two-way repeated measures ANOVA. With respect to locomotion, the mice with MCAO did not respond to the change of environment from light to dark, whereas mice in all other groups became hyperactive in response to the dark environment, either at 5 weeks (data not shown) or 7 weeks after the insult (Fig. 6B). With respect to rearing, there were significant group differences at both 5 weeks (data not shown) and 7 weeks (Fig. 6C) after the insult. The mice with HI exhibited significantly less rearing compared with mice in all other groups.

Discussion

In this study, we have demonstrated that permanent occlusion of the MCA in CB-17 mice induces a highly reproducible and selective cortical infarction. We believe that our model has clinical relevance to, at least a portion of infants with stroke, as an isolated large infarct in the vascular territory of left MCA is most commonly observed in infants with stroke (Lee et al., 2005; Sreenan et al., 2000). This high degree of consistency allows the effective screening of various experimental treatments using smaller numbers of animals. The most important point in achieving this high reproducibility is the use of the CB-17 strain, which exhibits very little variation in the cerebral vascular structure (Taguchi et al., 2010). It is known that the degree of brain damage and its reproducibility in neonatal rodent models of HI and stroke are dependent upon the strain used (Comi et al., 2005; Sheldon et al., 1998). In addition to the high reproducibility, the advantages of our model are its simple procedure and high long-term survival, which provides the opportunity for long-term evaluation of neuropathological and functional outcomes. Indeed, our model exhibited significant long-term neurofunctional deficits.

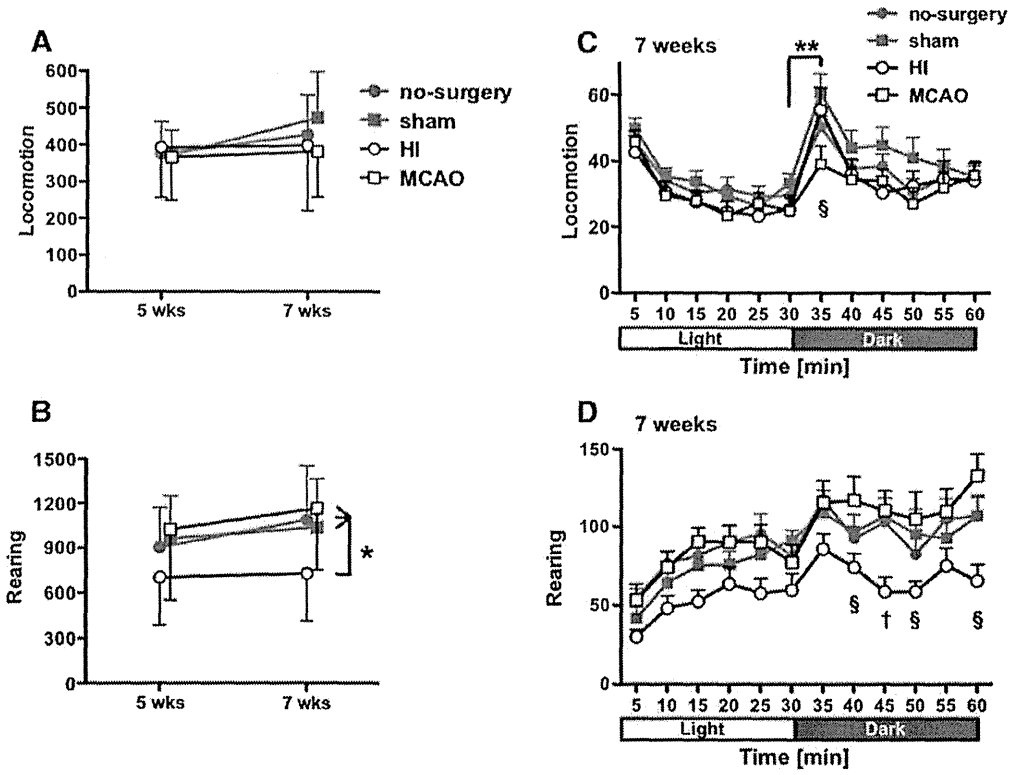

Fig. 6. Open-field test. (A, B) Overall activities during the 60-min session 5 and 7 weeks after the insult were analyzed by two-way repeated measures ANOVA. While there was no time difference with respect to either locomotion or rearing, there were significant group differences with respect to rearing, but not locomotion; mice with HI were significantly hypoactive compared with mice in the other three groups. (no-surgery $n = 14$; sham-surgery $n = 10$; HI $n = 16$; MCAO $n = 14$, 5 weeks after the insult. no-surgery $n = 13$; sham-surgery $n = 11$; HI $n = 16$; MCAO $n = 13$, 7 weeks after the insult). (C, D) Temporal changes in 5-min increments were analyzed by repeated-measures two-way ANOVA. There were significant group differences with respect to locomotion at 7 weeks after the insult. Mice in the MCAO group were significantly hypoactive during the first 5-min period in the dark than mice in the HI group. § $P < 0.05$. There were significant increases in the activity from the last 5-min period in the light environment to the first period in the dark environment in all groups except for the MCAO group. ** $P < 0.01$. With respect to rearing, there were significant group differences at 7 weeks. Mice in the HI group exhibited significantly less rearing activity. §$P < 0.05$, compared with MCAO group, †$P < 0.05$, compared with the no-surgery, sham-surgery and MCAO groups. Mean ± SEM.

Six models of neonatal stroke using artery obstruction have been developed (Ashwal et al., 1995, 2007; Bonnin et al., 2011; Comi et al., 2004; Derugin et al., 1998, 2000; Mitsufuji et al., 1996; Renolleau et al., 1998; Wen et al., 2004), and are summarized in Table 3. All models, except one, exhibit obvious inter-animal variability; some of the animals subjected to the insult do not develop infarct, as is the case in the HI model. In a permanent MCAO model developed by Wen et al. (2004), in which a tailor-made intraluminal suture embolus was placed in P7 SD rats, infarct was noted in all 10 pups that were subjected to the insult. However, the long-term survival was not reported. Taken together, among the currently available rodent models of neonatal stroke our model exhibits the highest reproducibility with excellent long-term survival. Nevertheless, those models, including ours, should be complementary, in order to lead to new understanding of the mechanisms of neonatal stroke and to find therapies for neonatal stroke. Our model has some weaknesses compared with other models. Firstly, this model does not utilize a reperfusion phase. Reperfusion may or may not occur in some patients, or the reperfusion may occur too late to activate its downstream events in other patients. Secondly, increasing or decreasing the degree of brain injury is not possible in this model. Thirdly, craniotomy results in stress to the animal and trauma to local tissues, even though the present study

Table 3
Immature rodent models of cerebral ischemia.

	Method of obstruction	Age and Species/strain	Ratio of infarct formation*	Long-term survival	Author and reference
1	t-f-MCAO	P14-18 or P10 SH rats	8/9	21% by 28 days	Ashwal et al., 1995, 2007
2	t-f-MCAO	P7 Sprague–Dawley rats	8/10, 20/31	71% by 7 days	Derugin et al., 1998, 2000
3	p-CCAO + t-CCAO†	P10 Wistar rats	NA	NA	Mitsufuji et al., 1996
4	p-MCAO + t-CCAO‡	P7 Wistar rats	10/10, 36/66	NA	Renolleau et al., 1998; Bonnin et al., 2011
5	p-CCAO	P12 CD1 mice	20/28	86% by 7 days	Comi et al., 2004
6	p-f-MCAO	P7 SD rats	10/10	NA	Wen et al., 2004
Present study	p-MCAO	P12 CB-17 mice	27/27	85% by 8 weeks	

These are unilateral cerebral ischemia models, unless otherwise noted. t-; transient. f-; intraluminal filament. p-; permanent. MCAO; middle cerebral artery occlusion. CCAO; common carotid artery occlusion. P; postnatal day. SH; spontaneously hypertensive. NA; not available. * Ratio of the number of animals presenting with obvious infarct to the number of animals that survived until the time of assessment. † Unilateral p-CCAO combined with contralateral t-CCAO. ‡ Unilateral MCAO by electrocoagulation combined with ipsilateral t-CCAO.

demonstrated that sham-surgery operated mice were not different from the no-surgery control mice, with respect to brain morphology, CBF, and behavior.

The differences in the variability between the two models (i.e., MCAO and HI) demonstrated in our study can provide insights into the mechanisms that lead to extensively variable susceptibility to HI insult by animals, even within littermates. The pivotal cause of the variation remains poorly understood. A number of explanations have been proposed for inter-animal variations in the extent of brain damage; 1) differences in collateral arteries in the brain (Rubino and Young, 1988), 2) the existence of several major MCA branching patterns (Rubino and Young, 1988), 3) subtle differences in the genetic background, 4) blood sugar level differences, which may result from variations in feeding times and amount (Chen et al., 2011; Hattori and Wasterlain, 1990), 5) temperature variation, 6) weight variation (Menzies et al., 1992), and 7) long surgery time and duration of isoflurane exposure (Chen et al., 2011). Our contrasting results in the two models suggest that these explanations are unlikely, because only the HI model exhibited substantial variability, despite the fact that all the aforementioned factors were consistent for both the MCAO and HI models. We cannot exclude the possibility that structural and physiological variations in the circle of Willis could contribute to the inconsistent brain damage after HI. Bonnin et al. (2011) reported that establishment of collateral recruitment via the basilar artery led to the presence or absence of a lesion. We also cannot exclude other possibilities, such as differences in the susceptibility to reperfusion damage, or in cardiovascular and respiratory function. As our model and the above-mentioned reproducible stroke model (Wen et al., 2004) are both permanent occlusion models, some mechanisms that occur during reperfusion may lead to large inter-animal variability.

There has only been one previous study in the literature that directly compared the MCAO and HI models (Ashwal et al., 2007). Unlike ours, variability in brain injury did not appear to be different between the two models in the previous study. The discrepancy between their results and ours may be due to the different MCAO procedures and the animals used. The previous report used a transient MCAO model in P10 spontaneously hypertensive rats, whereas we used a permanent MCAO model in P12 CB-17 mice.

We observed thalamic damage that was confined to the ipsilateral VPN in our MCAO model. As the VPN is supplied by thalamoperforating arteries originating from the basilar artery systems (Oscar and Holschneider, 2012), MCAO does not cause direct ischemic injury to this nucleus. Secondary neuronal damage in the thalamic nuclei after focal ischemia has been reported in adult rat models (Dihne et al., 2002; Schroeter et al., 2006). The damage in VPN was possibly due to retrograde degeneration of the thalamocortical projection (Dihne et al., 2002). Thalamic atrophy has been seen in children with neonatal MCA infarct (Giroud et al., 1995).

Our MCAO model exhibited neurological dysfunction in the rotarod and open-field tests; the mice with MCAO lost the response to a change of the environment from light to dark, while their overall activities were not disturbed significantly. The results in behavioral tests in immature rodent models of stroke or HI are not consistent and can often be contradictory. Rodents with ischemic insult exhibited significantly poorer rotarod performance compared with controls in some (Chen et al., 2012; Jansen and Low, 1996), but not all studies (Aden et al., 2003; Kadam et al., 2009; Lubics et al., 2005). Similarly, rodents with ischemic insult exhibited altered behavior in open-field test in some studies (Aden et al., 2002; Kadam et al., 2009; Lubics et al., 2005), but not in others (de Paula et al., 2009). The discrepancies among the reports may be due to differences in species/strain (de Visser et al., 2006), in the extent of brain damage, in the timing of the assessment (Lubics et al., 2005), and in the experimental paradigm. In the future more sensitive measures will be needed to confirm these results.

Seizure behavior, which is one of the main presenting symptoms in neonates with stroke, was not observed in our model during the 2-hour period following artery occlusion. Seizure behavior has been reported in a stroke model in immature CD1 mice (Comi et al., 2004), but not in other stroke models in immature rodents. That is likely due to strain-related differences in the susceptibility to seizures (Comi et al., 2005) or simply due to a lack of detailed assessment for seizure activities in the models. One possible reason to explain the inability to cause seizure in our model would be the distribution of the brain injury, which is confined to the ipsilateral cortex and did not involve the hippocampus. More detailed and longer observation periods will be needed before we can conclude that our model does not cause seizure activity, as the median time to seizure after the insult can be more than 2 h in some strains (Comi et al., 2005).

Conclusions

We believe that this model is useful for detailed analyses in preclinical studies of neonatal stroke using a smaller number of animals, because of its high reproducibility, excellent long-term survival rate, and measurable neurofunctional deficits, and that this model will be useful in assessing functional improvement in response to experimental therapies.

Disclosures

None.

Sources of funding

This work was funded by a Grant-in-Aid for Scientific Research (JSPS KAKENHI 24591617) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Acknowledgments

We thank Manami Sone for excellent technical assistance. We also thank Kenichi Mishima, Ph.D., Masafumi Ihara, M.D., and Kenichi Yamahara M.D. for helpful discussions.

References

Aden, U., Dahlberg, V., Fredholm, B.B., Lai, L.J., Chen, Z., Bjelke, B., 2002. MRI evaluation and functional assessment of brain injury after hypoxic ischemia in neonatal mice. Stroke 33, 1405–1410.

Aden, U., Halldner, L., Lagercrantz, H., Dalmau, I., Ledent, C., Fredholm, B.B., 2003. Aggravated brain damage after hypoxic ischemia in immature adenosine a2a knockout mice. Stroke 34, 739–744.

Ashwal, S., Cole, D.J., Osborne, S., Osborne, T.N., Pearce, W.J., 1995. A new model of neonatal stroke: reversible middle cerebral artery occlusion in the rat pup. Pediatr. Neurol. 12, 191–196.

Ashwal, S., Tone, B., Tian, H.R., Chong, S., Obenaus, A., 2007. Comparison of two neonatal ischemic injury models using magnetic resonance imaging. Pediatr. Res. 61, 9–14.

Bonnin, P., Leger, P.L., Deroide, N., Fau, S., Baud, O., Pocard, M., Charriaut-Marlangue, C., Renolleau, S., 2011. Impact of intracranial blood-flow redistribution on stroke size during ischemia-reperfusion in 7-day-old rats. J. Neurosci. Methods 198, 103–109.

Chabrier, S., Husson, B., Dinomais, M., Landrieu, P., Nguyen The Tich, S., 2011. New insights (and new interrogations) in perinatal arterial ischemic stroke. Thromb. Res. 127, 13–22.

Chen, H., Burris, M., Fajilan, A., Spagnoli, F., Tang, J., Zhang, J.H., 2011. Prolonged exposure to isoflurane ameliorates infarction severity in the rat pup model of neonatal hypoxia–ischemia. Transl. Stroke Res. 2, 382–390.

Chen, H., Spagnoli, F., Burris, M., Rolland, W.B., Fajilan, A., Dou, H., Tang, J., Zhang, J.H., 2012. Nanoerythropoietin is 10-times more effective than regular erythropoietin in neuroprotection in a neonatal rat model of hypoxia and ischemia. Stroke 43, 884–887.

Comi, A.M., Weisz, C.J., Highet, B.H., Johnston, M.V., Wilson, M.A., 2004. A new model of stroke and ischemic seizures in the immature mouse. Pediatr. Neurol. 31, 254–257.

Comi, A.M., Johnston, M.V., Wilson, M.A., 2005. Strain variability, injury distribution, and seizure onset in a mouse model of stroke in the immature brain. Dev. Neurosci. 27, 127–133.

Dammann, O., Ferriero, D., Gressens, P., 2011. Neonatal encephalopathy or hypoxic–ischemic encephalopathy? Appropriate terminology matters. Pediatr. Res. 70, 1–2.
de Paula, S., Vitola, A.S., Greggio, S., de Paula, D., Mello, P.B., Lubianca, J.M., Xavier, L.L., Fiori, H.H., Dacosta, J.C., 2009. Hemispheric brain injury and behavioral deficits induced by severe neonatal hypoxia–ischemia in rats are not attenuated by intravenous administration of human umbilical cord blood cells. Pediatr. Res. 65, 631–635.
de Visser, L., van den Bos, R., Kuurman, W.W., Kas, M.J., Spruijt, B.M., 2006. Novel approach to the behavioural characterization of inbred mice: automated home cage observations. Genes Brain Behav. 5, 458–466.
de Vries, L.S., Groenendaal, F., Eken, P., van Haastert, I.C., Rademaker, K.J., Meiners, L.C., 1997. Infarcts in the vascular distribution of the middle cerebral artery in preterm and fullterm infants. Neuropediatrics 28, 88–96.
Derugin, N., Ferriero, D.M., Vexler, Z.S., 1998. Neonatal reversible focal cerebral ischemia: a new model. Neurosci. Res. 32, 349–353.
Derugin, N., Wendland, M., Muramatsu, K., Roberts, T.P., Gregory, G., Ferriero, D.M., Vexler, Z.S., 2000. Evolution of brain injury after transient middle cerebral artery occlusion in neonatal rats. Stroke 31, 1752–1761.
Dihne, M., Grommes, C., Lutzenburg, M., Witte, O.W., Block, F., 2002. Different mechanisms of secondary neuronal damage in thalamic nuclei after focal cerebral ischemia in rats. Stroke 33, 3006–3011.
Gardner, M.A., Hills, N.K., Sidney, S., Johnston, S.C., Fullerton, H.J., 2010. The 5-year direct medical cost of neonatal and childhood stroke in a population-based cohort. Neurology 74, 372–378.
Giroud, M., Fayolle, H., Martin, D., Baudoin, N., Andre, N., Gouyon, J.B., Nivelon, J.L., Dumas, R., 1995. Late thalamic atrophy in infarction of the middle cerebral artery territory in neonates. A prospective clinical and radiological study in four children. Childs Nerv. Syst. 11, 133–136.
Golomb, M.R., Cvijanovich, N.Z., Ferriero, D.M., 2006. Neonatal brain injury, In: Swaiman, K.F., Ashwal, S., Ferriero, D.M. (Eds.), Pediatric neurology, principles & practice, 4th ed. Mosby Elsevier, Philadelphia, pp. 329–343.
Hagberg, H., Peebles, D., Mallard, C., 2002. Models of white matter injury: comparison of infectious, hypoxic–ischemic, and excitotoxic insults. Ment. Retard. Dev. Disabil. Res. Rev. 8, 30–38.
Hattori, H., Wasterlain, C.G., 1990. Posthypoxic glucose supplement reduces hypoxic–ischemic brain damage in the neonatal rat. Ann. Neurol. 28, 122–128.
Jansen, E.M., Low, W.C., 1996. Long-term effects of neonatal ischemic–hypoxic brain injury on sensorimotor and locomotor tasks in rats. Behav. Brain Res. 78, 189–194.
Johnston, M.V., Ferriero, D.M., Vannucci, S.J., Hagberg, H., 2005. Models of cerebral palsy: which ones are best? J. Child Neurol. 20, 984–987.
Kadam, S.D., Mulholland, J.D., Smith, D.R., Johnston, M.V., Comi, A.M., 2009. Chronic brain injury and behavioral impairments in a mouse model of term neonatal strokes. Behav. Brain Res. 197, 77–83.
Lee, J., Croen, L.A., Lindan, C., Nash, K.B., Yoshida, C.K., Ferriero, D.M., Barkovich, A.J., Wu, Y.W., 2005. Predictors of outcome in perinatal arterial stroke: a population-based study. Ann. Neurol. 58, 303–308.
Lubics, A., Reglodi, D., Tamas, A., Kiss, P., Szalai, M., Szalontay, L., Lengvari, I., 2005. Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic-ischemic injury. Behav. Brain Res. 157, 157–165.
Menzies, S.A., Hoff, J.T., Betz, A.L., 1992. Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model. Neurosurgery 31, 100–106.
Mitsufuji, N., Yoshioka, H., Okano, S., Nishiki, T., Sawada, T., 1996. A new model of transient cerebral ischemia in neonatal rats. J. Cereb. Blood Flow Metab. 16, 237–243.
Nelson, K.B., Lynch, J.K., 2004. Stroke in newborn infants. Lancet Neurol. 3, 150–158.
Ohshima, M., Tsuji, M., Taguchi, A., Kasahara, Y., Ikeda, T., 2012. Cerebral blood flow during reperfusion predicts later brain damage in a mouse and a rat model of neonatal hypoxic-ischemic encephalopathy. Exp. Neurol. 233, 481–489.
Oscar, U., Holschneider, D.P., 2012. Vascular supply. In: Watson, C., Paxinos, G., Puelles, L. (Eds.), The Mouse Nervous System. Academic Press, London, pp. 459–472.
Renolleau, S., Aggoun-Zouaoui, D., Ben-Ari, Y., Charriaut-Marlangue, C., 1998. A model of transient unilateral focal ischemia with reperfusion in the p7 neonatal rat: morphological changes indicative of apoptosis. Stroke 29, 1454–1460.
Rice III, J.E., Vannucci, R.C., Brierley, J.B., 1981. The influence of immaturity on hypoxic–ischemic brain damage in the rat. Ann. Neurol. 9, 131–141.
Rubino, G.J., Young, W., 1988. Ischemic cortical lesions after permanent occlusion of individual middle cerebral artery branches in rats. Stroke 19, 870–877.
Schroeter, M., Zickler, P., Denhardt, D.T., Hartung, H.P., Jander, S., 2006. Increased thalamic neurodegeneration following ischaemic cortical stroke in osteopontin-deficient mice. Brain 129, 1426–1437.
Sheldon, R.A., Sedik, C., Ferriero, D.M., 1998. Strain-related brain injury in neonatal mice subjected to hypoxia–ischemia. Brain Res. 810, 114–122.
Sreenan, C., Bhargava, R., Robertson, C.M., 2000. Cerebral infarction in the term newborn: clinical presentation and long-term outcome. J. Pediatr. 137, 351–355.
Taguchi, A., Soma, T., Tanaka, H., Kanda, T., Nishimura, H., Yoshikawa, H., Tsukamoto, Y., Iso, H., Fujimori, Y., Stern, D.M., Naritomi, H., Matsuyama, T., 2004. Administration of $CD34^+$ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J. Clin. Invest. 114, 330–338.
Taguchi, A., Kasahara, Y., Nakagomi, T., Stern, D.M., Fukunaga, M., Ishikawa, M., Matsuyama, T., 2010. A reproducible and simple model of permanent cerebral ischemia in CB-17 and SCID mice. J. Exp. Stroke Transl. Med. 3, 28–33.
Tsuji, M., Wilson, M.A., Lange, M.S., Johnston, M.V., 2004. Minocycline worsens hypoxic-ischemic brain injury in a neonatal mouse model. Exp. Neurol. 189, 58–65.
Tsuji, M., Taguchi, A., Ohshima, M., Kasahara, Y., Ikeda, T., 2012. Progesterone and allopregnanolone exacerbate hypoxic-ischemic brain injury in immature rats. Exp. Neurol. 233, 214–220.
Volpe, J.J., 2012. Neonatal encephalopathy: an inadequate term for hypoxic–ischemic encephalopathy. Ann. Neurol. 72, 156–166.
Wen, T.C., Rogido, M., Gressens, P., Sola, A., 2004. A reproducible experimental model of focal cerebral ischemia in the neonatal rat. Brain Res. Brain Res. Protoc. 13, 76–83.

Letter to the Editor

Stroke welcomes Letters to the Editor and will publish them, if suitable, as space permits. Letters must reference a Stroke *published-ahead-of-print article or an article printed within the past 3 weeks. The maximum length is 750 words including no more than 5 references and 3 authors. Please submit letters typed double-spaced. Letters may be shortened or edited.*

Letter by Taguchi et al Regarding Article, "Granulocyte Colony-Stimulating Factor in Patients With Acute Ischemic Stroke: Results of the AX200 for Ischemic Stroke Trial"

To the Editor:

We read with interest a recent article by Ringelstein et al.[1] The authors described the results of a phase 2B clinical trial of granulocyte colony-stimulating factor (G-CSF) for patients with stroke, which did not result in any beneficial effects. The authors concluded that the failure of this clinical trial was mainly attributable to the problem of translating findings from the animal laboratory to patients with clinical stroke and thus raised the question, "Are rodent models questionable for predicting stroke drug efficacy in humans?" However, we think that the negative results of this clinical trial had been predictable from previous results of basic experiments with animal models.

The clinical trial was designed on the basis of a meta-analysis of experimental models of cerebral ischemia.[2] However, in the majority of cases, the experiments that were included were of transient cerebral ischemia ranging from 1 to 3 hours. Transient ischemia is a model of ischemia-reperfusion injury, and the therapeutic target is mainly protection from apoptotic neural cell death caused by oxidative stress after reperfusion. In contrast, most cases of human stroke show massive necrotic neural cell death caused by a poor supply of cerebral blood flow. Although, in the end, neural cell death is observed in both types of ischemia, the actual pathological states and therapeutic targets are different from each other, and the discrepancy between apoptotic and necrotic cell death should be considered when the clinical trial is designed. We point out that we clearly demonstrated that G-CSF has a negative effect on stroke outcome using a permanent cerebral artery ligation model in immunocompetent mice, and we drew attention to the discrepancy between transient and permanent cerebral occlusion models.[3] Furthermore, activated granulocyte, which is significantly mobilized by G-CSF from bone marrow to peripheral blood, is well known to enhance brain damage in experimental stroke model through enhancing inflammation at the site of cerebral ischemia.[4] Therefore, it is not surprising that the results of the clinical study show that G-CSF has no therapeutic effect on the outcome of patients with stroke, and the results are not dissimilar to previous findings shown in an experimental stroke model.

In conclusion, we think that it is not rational to conclude that rodent models are questionable in predicting stroke drug efficacy in humans. With regard to the clinical trial in question, previous findings obtained by basic experiments had predicted the negative effect of G-CSF on stroke outcomes (ie, other than experiments with transient ischemia models that mimic patients who had reperfusion injury after recanalization; however, this is not the case with majority of patients with stroke). We think that rodent models are useful for human trials as long as the therapeutic target and the patients enrolled are given proper consideration when the clinical trial is first designed.

Disclosures

None.

Akihiko Taguchi, MD
Yukiko Kasahara, PhD
Department of Regenerative Medicine Research
Institute of Biomedical Research and Innovation
Hyogo, Japan

Tomohiro Matsuyama, MD
Laboratory of Neurogenesis and CNS Repair
Institute for Advanced Medical Sciences
Hyogo College of Medicine
Hyogo, Japan

1. Ringelstein EB, Thijs V, Norrving B, Chamorro A, Aichner F, Grond M, et al. AXIS 2 Investigators. Granulocyte colony-stimulating factor in patients with acute ischemic stroke: results of the AX200 for Ischemic Stroke trial. *Stroke*. 2013;44:2681–2687.
2. Minnerup J, Heidrich J, Wellmann J, Rogalewski A, Schneider A, Schäbitz WR. Meta-analysis of the efficacy of granulocyte-colony stimulating factor in animal models of focal cerebral ischemia. *Stroke*. 2008;39:1855–1861.
3. Taguchi A, Wen Z, Myojin K, Yoshihara T, Nakagomi T, Nakayama D, et al. Granulocyte colony-stimulating factor has a negative effect on stroke outcome in a murine model. *Eur J Neurosci*. 2007;26:126–133.
4. Justicia C, Panés J, Solé S, Cervera A, Deulofeu R, Chamorro A, et al. Neutrophil infiltration increases matrix metalloproteinase-9 in the ischemic brain after occlusion/reperfusion of the middle cerebral artery in rats. *J Cereb Blood Flow Metab*. 2003;23:1430–1440.

(*Stroke*. 2014;45:e8.)

***Stroke* is available at http://stroke.ahajournals.org** **DOI: 10.1161/STROKEAHA.113.003683**

Stroke
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Letter by Taguchi et al Regarding Article, "Granulocyte Colony-Stimulating Factor in Patients With Acute Ischemic Stroke: Results of the AX200 for Ischemic Stroke Trial"

Akihiko Taguchi, Yukiko Kasahara and Tomohiro Matsuyama

Stroke. 2014;45:e8; originally published online December 10, 2013;
doi: 10.1161/STROKEAHA.113.003683
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0039-2499. Online ISSN: 1524-4628