$CD133^+$ cells from human umbilical cord blood reduce cortical damage and promote axonal growth in neonatal rat organ co-cultures exposed to hypoxia

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Abbreviations

HIE, hypoxic–ischemic encephalopathy

EPC, endothelial progenitor cell

PBS, phosphate-buffered saline

DiI, 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate

hMit, human mitochondria

IB4, isolectin B4

DAPI, 4’,6’-diamidino-2-phenylindole

Running Title

CD133^{+} cells reduce hypoxic brain insult in neonatal rat

Abstract

To evaluate the effect of $CD133^+$ cells (endothelial progenitor cells) on the hypoxia-induced suppression of axonal growth of cortical neurons and the destruction of blood vessels (endothelial cells), we used anterograde axonal tracing and immunofluorescence in organ co-cultures of the cortex and the spinal cord from 3-day-old neonatal rats. $CD133^+$ cells prepared from human umbilical cord blood were added to the organ co-cultures after hypoxic insult, and axonal growth, vascular damage and apoptosis were evaluated. Anterograde axonal tracing with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate was used to analyze axonal projections from the cortex to the spinal cord. Immunolabeling co-cultured tissues of the cortex and the spinal cord were used to investigate the effect of $CD133^+$cells on the survival of blood vessels and apoptosis in the brain cortex. Hypoxia remarkably suppressed axonal growth in organ co-cultures of the cortex and the spinal cord, and this suppression was significantly restored by the addition of $CD133^+$ cells. $CD133^+$ cells also reduced the hypoxia-induced destruction of the cortical blood vessels and apoptosis. $CD133^+$ cells had protective effects on hypoxia-induced injury of neurons and blood vessels of the brain cortex *in vitro*. These results suggest that $CD133^+$ cell transplantation may be a possible therapeutic intervention for perinatal

hypoxia-induced brain injury.

KEY WORDS

Brain damage; CD133; Hypoxia; Neonatal rat; Slice culture

1. Introduction

Hypoxic–ischemic encephalopathy (HIE) is a major cause of acute mortality and neurological complications in the newborn. However, its pathogenesis has not been fully elucidated. This type of perinatal injury results in motor disorders accompanied by other functional disturbances (Volpe, 2001). The cerebral cortex is one of the regions in which hypoxic damage is primarily observed (Grafe, 1994; Rice et al., 1981; Stadlin et al., 2003). Although many interventions for perinatal hypoxia-induced brain injury including HIE have been attempted, few have become established therapies. Therefore, the development of new strategies for treating perinatal hypoxia-induced brain injury is urgently needed.

In recent years stem cell therapies have been reported in several fields of medicine. Endothelial progenitor cells (EPC) are one of the major stem/progenitor cell subsets with the potential for repairing vascular injury. EPCs from adult human peripheral blood were initially described by Asahara et al. (Asahara et al., 1997). CD133 is a hematopoietic stem cell marker which is expressed on EPCs, but not on mature vascular endothelial cells (Yin et al., 1997). EPCs are involved in promoting neovascularization and also have the capacity to protect and repair ischemic lesions (Asahara et al., 1997; van Velthoven et al., 2009). Since these functions are central to rescuing ischemic tissue,

these EPCs have been the focus of regenerative medicine for ischemia. More recently, Murohara et al. reported that EPCs derived from umbilical cord blood have a higher regenerative potential than adult bone marrow-derived EPCs (Murohara et al., 2000). Since umbilical cord blood can be easily obtained without harm to either mother or infant, and is therefore a readily available source of cells for autotransplantation, EPCs from umbilical cord blood have an additional advantage for use as a therapy for infants. Moreover, a recent report has suggested that the transplantation of umbilical cord-derived EPCs improved ischemic brain damage and promoted neurogenesis in a model of adult stroke in mice (Taguchi et al., 2004). Therefore, we speculated that the transplantation of $CD133^{+}$ cells, as a EPCs containing fraction, from umbilical cord blood could also be a useful therapy in perinatal hypoxia-induced brain injury.

Oishi et al. have established a novel *in vitro* assay system for axonal regeneration in the central nervous system using organ co-cultures of the brain motor cortex and the spinal cord from neonatal rats (Oishi et al. 2004). Previous studies using this system in normoxic condition have shown that corticospinal tract axonal growth was promoted by the addition of stem/progenitor cells (Kamei et al., 2004; Kamei et al., 2007; Yamamoto et al., 2008). Additionally, our preliminary data have shown that axonal growth in these neonatal rat organ co-cultures correlated with the degree of hypoxic-ischemic damage in

the cortical slices. The purpose of this study was to assess the effects of co-culturing $CD133^+$ cells from human umbilical cord blood on neurons and cortical blood vessels damaged by hypoxia using this novel *in vitro* system.

2. Experimental procedures

2.1. Animals

This study was carried out in accordance with Guideline from the Committee of Research Facilities for Laboratory Animal Science at Hiroshima University. We used 3 d old unsexed Sprague–Dawley rats.

2.2. Isolation and preparation of human $CD133^+$ cells

The protocol used was approved by the Hiroshima University Research Ethics Committee, Japan, and informed consent was obtained from all participants. Human $CD133^+$ cells were prepared from freshly obtained human umbilical cord blood according to our previous report (Yamamoto et al., 2008). Umbilical cord blood mononuclear cells were isolated by density-gradient centrifugation of buffy coats using Histopaque 1077 (Sigma-Aldrich, Oakville, Canada). $CD133^+$ cells were purified from the mononuclear cells using anti-CD133 monoclonal antibody-conjugated microbeads

(CD133 MicroBead Kit; Miltenyi Biotec, Bergisch-Gladback, Germany) and a magnetically activated cell sorter (auto-MACS; Miltenyi Biotec) following the manufacturer's protocol. To confirm the purity of $CD133^+$ cells, the enriched $CD133^+$ cells were used in cytometric analyses using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo (TreeStar, Inc, Ashland, OR, USA). The isolated cells contained at least 70% purity $CD133^+CD34^+$ cells (Fig. 1) (Yamamoto et al., 2008). The isolated $CD133^+$ cells were resuspended with freezing medium (CELLBANKER; Zenoaq, Fukushima, Japan) and were cryopreserved until use.

2.3. Organ co-cultures

Organ co-cultures of brain cortices and thoracic spinal cords from 3 d old rats were prepared as reported previously (Kamei et al., 2004; Oishi et al., 2004; Yamamoto et al., 2008). The brains were cut in the coronal plane to produce 400 μm sections using a Vibratome (Dosaka EM, Kyoto, Japan), and the motor cortex was dissected from each side of two or three coronal sections. The first sequential coronal slice was cut about 2.5 mm from the frontal pole. The thoracic spinal cords were bisected in the sagittal plane using a razor blade. The dissected pieces of cortex and spinal cord were placed on membranes (Millicell-CM; Millipore, Billerica, MA, USA) in 1 ml culture medium,

composed of 50% Basal Medium Eagle with Earle's Salts (Sigma, St Louis, MO, USA), 25% heat-inactivated horse serum (Gibco, Grand Island, NY, USA), 25% Earle's Balanced Salt Solution (Sigma), 1 mM L-glutamine and 0.5% D-glucose, in six-well tissue culture plates (Greiner bio-one, Kremsmünster, Austria). The cultures were incubated for 24 h, and the following day, the spinal cord fragments were placed next to the cortical white matter (Fig. 2A). The co-cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37ºC and the medium was replaced every 3 or 4 d. The co-cultures were incubated for up to 14 d for tracing axonal growth and for up to 4 or 6 d for immunolabeling experiments.

2.4. Hypoxic stress and the effect of CD133$^+$ cells

To investigate the effect of the duration of hypoxic exposure, on day 2 in culture, the organ co-cultures were transferred to a hypoxic chamber, maintained at 2% O_2 balanced with N_2 (MCO-5M (UV); SANYO, Osaka, Japan), immediately after the cortical and the spinal cord tissues had been placed in contact with each other. Four groups of co-cultures were set up, three of which were exposed to hypoxic conditions for 24, 48 or 72 h while the fourth control group (normoxic control group) was maintained in 20% O_2.

The effect of $CD133^+$ cells on hypoxia-induced cortical damage was assessed using three groups of co-cultures: a first hypoxic group which had been incubated in 2% O_2 for 24 h as described above (hypoxic control group), a second hypoxia-treated group to which CD 133^+ cells were added after hypoxia exposure (CD133 group), and a non-hypoxic group (non-hypoxic control group) which had only been incubated in 20% O_2. In the CD133 group, 1×10^4 viable enriched $CD133^+$ cells in 2 µl phosphate-buffered saline (PBS; Sigma) were added to each culture on day 3 onto the membrane around edge of the brain cortex, immediately after exposure to hypoxia for 24 h, while 2 µl PBS were added to the co-cultures in the non-hypoxic control and the hypoxic control groups. Cryopreserved $CD133^+$ cells were thawed and resuspended with PBS, and cell viability was determined using an aliquot of $CD133^+$ cells staining with trypan blue (Nacalai Tesque, Kyoto, Japan) immediately before addition to the co-cultures.

2.5. Tracing axonal growth

Axonal projections from the cortex to the spinal cord were labeled in the co-cultures with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA) for anterograde axonal tracing. The co-cultures were fixed for 7 d in 4% paraformaldehyde at 4ºC. Small crystals of DiI were placed

into the center of horizontal axis and vertical axis of each cortical slice culture (Fig. 2A), and the co-cultures were incubated for another 14 d in 0.1M phosphate buffer in a humidified, 5% CO_2 atmosphere at 37ºC. The co-cultures were mounted on glass slides and covered with Vectashield (Vector, Burlingame, CA, USA) and glass coverslips. To analyze axonal growth, we counted the number of labeled axons passing through reference lines running parallel to the junction between the cortex and the spinal cord, at distances of 500 and 1000 μm from the junction (Fig. 2B). We evaluated axonal growth using images that were taken at ×200 magnification, at different focal planes, and all fibers crossing the reference line were counted.

2.6. Immunolabeling co-cultured tissues

The co-cultured tissues in the non-hypoxic control, hypoxic control and CD133 groups were stained, 3 days after the addition of PBS with or without CD 133^+ cells, as previously described (Park et al., 2004; Yamamoto et al., 2008). The tissues were incubated with mouse anti-human mitochondria (hMit) monoclonal antibody (1: 200, Chemicon, Billerica, MA, USA) at 4ºC overnight. The following day, the tissues were incubated with Alexa Fluor 594-conjugated goat anti-mouse antibodies (1: 500, Invitrogen, Carlsbad, CA, USA) and fluorescein isothiocyanate-conjugated isolectin B4

(IB4; 1:100, Vector) at 4ºC overnight. On the third day, the tissues were counterstained with 4',6'-diamidino-2-phenylindole (DAPI; 1:1000, Vector), and observed under a fluorescence microscope (Keyence, Osaka, Japan). The location of the $CD133^+$ cells was identified from the hMit staining. The structure of cortical blood vessels was also assessed in IB4-stained sections (Ernst and Christie, 2006) and the effects of hypoxia and of the addition of $CD133^+$ cells on these vessels were assessed in all layers in z-stacked images to which 3-dimensional (3D) deconvolution was applied. The total length of the vessels in the center of each cortical slice in an area of 730μm × 550 μm, which is a region similar to where DiI crystal was placed in other slice, was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

For evaluation of apoptosis in the cortical cultures, the tissues were stained with caspase-3 one day after the addition of PBS with or without CD 133^+ cells (Nakajima et al., 2000). The tissues were incubated with anti-caspase-3 antibody (1:200, Cell Signaling Technology, Beverly, MA, USA) at room temperature overnight. The following day, the tissues were incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibodies (1: 500, Invitrogen) at 4ºC overnight. On the third day, the tissues were counterstained with DAPI, and observed. The effects of hypoxia and of $CD133^+$ cells on apoptosis were assessed. The apoptotic cells in the unit area were counted.

2.7. Statistical analysis

Results were expressed as means ± standard errors. The statistical differences between two groups were assessed using the Mann-Whitney U test. The Dunnett's test was used to compare parameters between three or four groups, using the normoxic control or the hypoxic control groups as a reference group. *P* values < 0.05 were considered to be statistically significant.

3. Results

3.1. Corticospinal axonal growth in organotypic co-cultures

We first evaluated axonal growth in the normoxic control and hypoxia-exposed groups to assess the effect of hypoxic damage in these organ co-cultures. The number of axons extending 500 μm in the normoxic control group was 3.9 ± 0.7 (n = 36). In contrast, the numbers in the groups exposed to hypoxia for 24, 48 or 72 h were 1.3 ± 0.5 (n = 18), 0 (n = 9) and 0 (n = 9), respectively (Fig. 3). This showed a significant reduction ($p < 0.05$) in the axonal growth to 500 μm from the cortex-spinal cord junction after exposure to hypoxic stress at 2% O_2 even for 24 h. The number of axons extending to 1000 μm in the 24 h hypoxia-exposed group was 0.3 ± 0.2 (n = 18) which was lower,

but not significantly lower, compared to 1.2 ± 0.3 (n = 36) in the normoxic control group. Exposure to hypoxia for 24 h reduced the mean number of axonal projection to the spinal cord in the co-cultures, but did not completely abolish them, at both reference points. Since we had shown that axonal growth impairment after 24 h in 2% O_2 could be evaluated quantitatively, we performed subsequent experiments under these conditions.

We next investigated the effect of adding $CD133^+$ cells to the co-cultures on axonal growth from the cortex to the spinal cord after exposure to hypoxia. In the hypoxic control group, either none or few short axons labeled with DiI were detectable in the spinal cord. In the CD133 group, however, more, longer axons were visible (Fig. 4); the number of axons extending to 500 µm in the CD133 group was 4.3 ± 1.4 (n = 9), which was significantly greater than in the hypoxic control group, in which the number of axons of similar length was 1.1 ± 0.8 (n = 9; $p = 0.037$). The number of axons extending to 1000 µm was also higher in the CD133 group (1.3 ± 0.7 versus 0.2 ± 0.2 in the hypoxic control group) but the difference was not significant ($p = 0.072$) (Fig. 5).

3.2. Location of $CD133^+$ cells in co-cultures

To assess the number and location of adding $CD133^+$ cells surviving after three days in the co-cultures, we used hMit-labeling to identify the $CD133^+$ cells. In the CD133 group,

roughly 10 hMit positive cells were observed per co-culture. The majority of these cells were scattered at the margins of the co-cultured tissues. There were no merged cells with hMit- and IB4-immunofluorescence nor were any hMit positive cells integrated into blood vessels (Fig. 6).

3.3. Blood vessels in the brain cortex

Co-cultures were immunolabeled with IB4 to investigate the effect of $CD133^{+}$cells on the survival of vascular endothelial cells. In the non-hypoxic control group, blood vessels had a regular morphology and had smooth vessel walls (Fig. 7A). In the hypoxic control and the CD133 groups, in which co-cultures had been exposed to hypoxia, the blood vessels were disrupted and the walls were jagged (Fig. 7B-C). The total length of the vessels per unit area were 8.7 ± 0.2 mm, 4.0 ± 0.8 mm and 5.6 ± 0.7 mm in the non-hypoxic control, hypoxic control and CD133 groups, respectively (n = 5 in each group) (Fig. 8). There were significantly fewer blood vessels in the hypoxic control group compared to the non-hypoxic control group ($p < 0.05$), and the CD133 group ($p < 0.05$).

3.4. Apoptosis in the brain cortex

Co-cultures were immunolabeled with caspase-3 to investigate the effect of $CD133^+$cells on apoptosis. In either the hypoxic control group or the CD133 group, total numbers of nuclei decreased and the numbers of small condensed nuclei increased compared to those in the non-hypoxic control group (Fig. 9). There were significantly more apoptotic cells in the hypoxic control group compared to the non-hypoxic control group ($p < 0.05$), and the CD133 group ($p < 0.05$) (Fig. 9). Caspase-3^+ cells per unit area were 1.0 ± 0.6, 7.6 ± 0.7 mm and 4.9 ± 2.5 mm in the non-hypoxic control (n = 3), hypoxic control (n = 13) and CD133 groups (n = 10), respectively (Fig. 10).

4. Discussion

Brain cortex-spinal cord organ co-culture is an established system that is a very useful *in vitro* model for assessing axonal growth in the central nervous system (Kamei et al., 2004; Kamei et al., 2007; Oishi et al., 2004; Yamamoto et al., 2008). In this system, agents that might affect axonal growth can be easily delivered at known concentrations, and their effects assessed. The effects of adding human cells to the co-cultures can be evaluated without having to consider graft-host interactions. Neural progenitor cells (Kamei et al., 2004), bone marrow stromal cells (Kamei et al., 2007) and $CD133^+$ cells derived from peripheral blood (Yamamoto et al., 2008) have been shown to enhance

axonal growth in normoxic condition. We have applied this *in vitro* assay system to the study of perinatal hypoxia-induced brain injury, and we demonstrated that 24-h hypoxic stress significantly and appropriately reduced axonal growth in the co-cultures from neonatal rats. We then showed that the addition of $CD133^+$ cells to the co-cultures improved corticospinal axonal growth after hypoxia-induced suppression. Our previous data have shown that axonal growth enhanced by addition of human $CD133^+$ cells even in normoxic condition (Yamamoto et al., 2008). And apoptotic cells in the cortical tissues exposed to hypoxia were also reduced by the addition of $CD133^+$ cells. It is therefore possible that the improvement in axonal length and number was due to direct effect on axonal growth and also due to inhibition of cell death in the culture. Interestingly, the addition of $CD133^+$ cells also reduced hypoxia-induced destruction of the cortical blood vessels. In an animal model of adult stroke, EPC transplantation has also shown potential as a therapeutic strategy (Taguchi et al., 2004). As for perinatal hypoxia-induced brain injury, to date whole human cord blood mononuclear cells (Meier et al., 2006) or stem/progenitor cells such as neuronal progenitor cells (Sato et al., 2008) and astrocytic stem cells (Zheng et al., 2006) have been reported to be potential therapeutic agents. However, we are not aware of any previous report describing the efficacy of EPCs.

Although Taguchi et al. (Taguchi et al., 2004) showed that EPC transplantation after stroke enhanced angiogenesis followed by neurogenesis, and promoted recovery, the mechanism mediating the beneficial effects of EPC transplantation is not clear at present. Possible mechanisms include the replacement of damaged cells by differentiated transplanted cells, cell-to-cell communication (Deregibus et al., 2007) involving the secretion of growth factors and cytokines, or the other effects. The data presented here showed that the number of hMit positive cells, indicating the $CD133^+$ human cells present in the co-cultures, was too small for them to have replaced the damaged tissue, and they were located only at the margin of the tissue. Moreover, this and our previous data indicated that $CD133^+$ cells have not differentiated into vascular endothelial cells in this system (Yamamoto et al., 2008). These suggest that the $CD133^+$ cells participated in the promotion of axonal growth and cell survival indirectly. The effects of $CD133^+$ cell can be attributed to some growth factors they secrete or cell-to-cell communication between $CD133^+$ cells and other cells, including endothelial cells, in the co-cultured tissues. Most of surviving $CD133^+$ cells may not have adhered to the co-cultures but remained on the membranes, and these cells also secreted factors. In addition, our preliminary data have confirmed that addition of human mononuclear cells did not affect axonal growth in our co-culture system. These findings indicate that

1×10^4 of $CD133^+$ cells are sufficient for trophic effect in our small organ cultures and that enhancing effect of axonal growth is distinctive of $CD133^+$ cells.

EPCs have been reported to secrete several growth factors including vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1) (Majka et al., 2001), hepatocyte growth factor (HGF), and granulocyte-colony stimulating factor (G-CSF) (Rehman et al., 2003). These factors, as well as any factors as yet undiscovered, may promote cell survival and axonal growth (Byrne et al., 2005; Silverman et al., 1999; Wong et al., 1997; Yang et al., 1998). The addition of $CD133^+$ cells to these co-cultures also reduced hypoxia-induced damage of the cortical blood vessels. Since survived cortical vascular endothelial cells also secrete neuroprotective factors (Guo et al., 2008; Lok et al., 2009), these factors can additionally protect neurons and facilitate axonal growth. van Velthoven et al. (van Velthoven et al., 2009) have proposed that the effects of early stem cell transplantation may be represented as neuroprotection rather than neuronal regeneration. They also suggested that most of the beneficial effects of transplanted cells are mediated by the production of various neurotrophic factors. If these suggestions are applicable to EPCs, we have demonstrated the most important effects of EPCs in perinatal hypoxia in the cortex.

In conclusions, $CD133^+$ cells had protective effects on neurons and vascular

endothelial cells exposed to hypoxia in the neonatal rat organ co-cultures of the cortex and the spinal cord. Although further studies are required to elucidate the precise mechanisms underlying the effect of $CD133^{+}$ cell on perinatal hypoxia-induced brain damage and to assess their application to *in vivo* animal models, our results suggest the possibility of a novel treatment for perinatal hypoxic brain injury.

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FIGURE LEGENDS

Figure 1. Representative FACS analysis of magnetically isolated $CD133^+$ cells derived from human umbilical cord mononuclear cells. The isolated cells consisted of over 70% $CD133^+CD34^+$ cells.

Figure 2. The organ co-culture of the brain motor cortex and the spinal cord from neonatal rats. (A) Photomicrograph of an organ co-culture involving a slice of brain cortex and a bisected spinal cord. The arrow shows the junction between the cortex and the spinal cord. * : Center of horizontal axis and vertical axis in the cortical slice culture where DiI crystals were inserted and blood vessels in all layers were observed using Z stack scan. (B) Schematic drawing of axonal projections from the cortex to the spinal cord and the reference lines used. Scale bar = 500 μm.

Figure 3. The quantitative assessment of axonal growth from the cortex to the spinal cord after hypoxic stress. The number of axons extending 500 μm from the cortex-spinal cord junction was significantly reduced after exposure to 2% O_2 even for 24 h (black; n = 18) compared to axons in the normoxic control group, unexposed to hypoxia (white; n = 36). No axons in the groups exposed to hypoxia for 48 and 72 h

extended to 500 μm (n = 9 in each group). The number of axons extending 1000 μm from the junction in the 24 h-hypoxic exposure group was also lower than the normoxic control group, but not significantly so. * $p < 0.05$.

Figure 4. The effect of $CD133^+$ cells on axonal growth from cortical tissue damaged by hypoxia. The arrowheads show the cortex-spinal cord junction and the arrows show projecting axons stained with DiI. More projecting axons were observed in the CD133 group, exposed to hypoxia before the addition of $CD133^+$ cells (B) than the hypoxic control group, which were only exposed to hypoxia (A). Scale bar = 500 μm.

Figure 5. The effect of $CD133^+$ cells on axonal growth from hypoxic damaged cortex. In the CD133 group, exposed to hypoxia before the addition of $CD133^+$ cells (gray; n = 9), the number of axons extending to 500 μm was significantly greater than the hypoxic control group, which were only exposed to hypoxia (black; n = 9; $p = 0.037$). The number of axons extending to 1000 μm from the junction also tended to be higher in co-cultures to which $CD133^+$cells had been added ($p = 0.072$). * $p < 0.05$.

Figure 6. Localization of hMit positive human umbilical cord blood-derived $CD133^+$

cells in co-cultures. hMit positive cells (red) (A) with nuclei counterstained with DAPI (blue) (B) were scattered at the margin of the cortical tissue (D). IB4-labeled blood vessels (green) (C) were rarely seen near the margins. There was no obvious merged cell with hMit- and IB4-immunofluorescence (D). Scale bar = 100 μm.

Figure 7. The effect of $CD133^+$ cells on the survival of endothelial cells. (A) In the non-hypoxic control (no hypoxia) group, blood vessels were morphologically regular. (B) In the hypoxic control (hypoxia only) group and (C) in the CD133 (hypoxia followed by $CD133^+$ cell addition) group, blood vessels were disrupted. However, more vessels were present in the CD133 group than the hypoxic control group. Scale bar = 100 μm.

Figure 8. Quantitative assessment of the effect of $CD133^+$ cells on endothelial cell survival after hypoxic stress. In the hypoxic control group (hypoxia only; black), the total length of the blood vessels per unit area was significantly lower than in the non-hypoxic control group (no hypoxia; white) and in the CD133 group (hypoxia followed by $CD133^+$ cell addition; gray) (n = 5 in each group). * $p < 0.05$.

Figure 9. The effect of $CD133^+$ cells on apoptosis of the cells in the brain cortex. (A) In the non-hypoxic control (no hypoxia) group, caspase-3 positive cell were hardly observed. (B) In the hypoxic control (hypoxia only) group and (C) in the CD133 (hypoxia followed by $CD133^+$ cell addition) group, total numbers of nuclei (blue) decreased and the numbers of small condensed nuclei increased compared to those in the non-hypoxic control (Fig. 9). However, caspase-3 positive cells (green) were reduced in the CD133 group than the hypoxic control group. Scale bar = 100 μm.

Figure 10. Quantitative assessment of the effect of $CD133^+$ cells on apoptosis of the cells in the brain cortex after hypoxic stress. In the hypoxic control group (hypoxia only; black), apoptotic cells per unit area was significantly more than in the non-hypoxic control group (no hypoxia; white) and in the CD133 group (hypoxia followed by $CD133^+$ cell addition; gray) (n = 5 in each group). * $p < 0.05$.

Fig 1

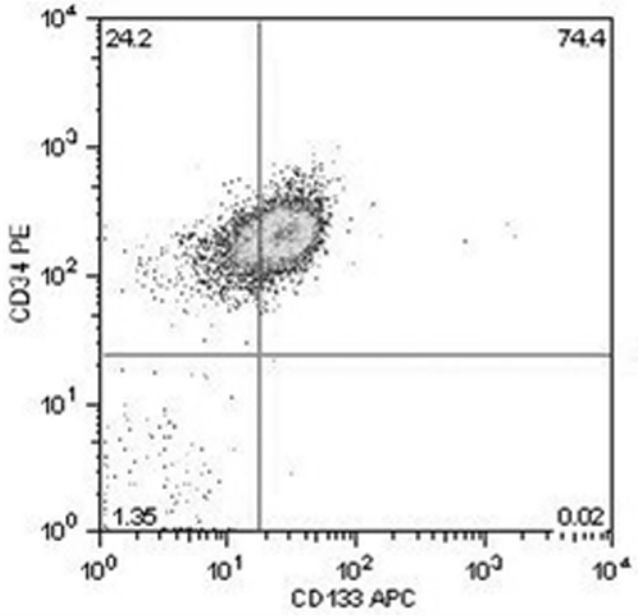
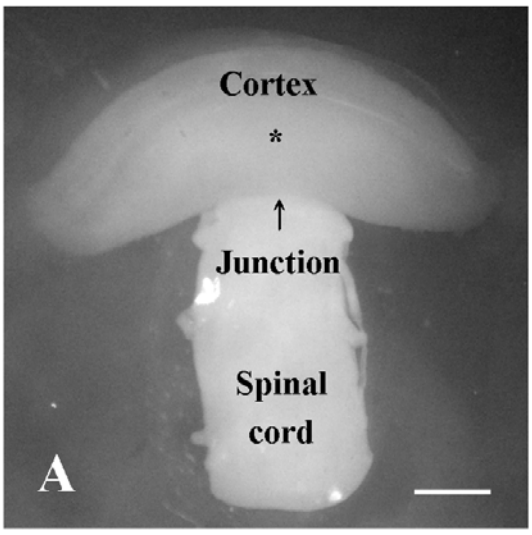

24.2
74.4
1.35
0.02
CD34 PE
CD133 APC

Fig 2


Cortex
*
Junction
Spinal
cord
A

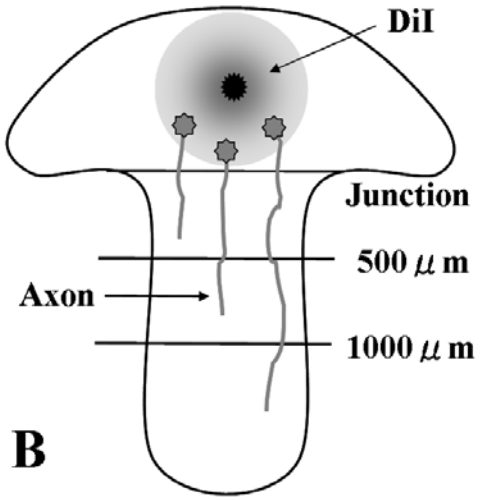

DiI
Junction
500 μm
Axon
1000 μm
B

Fig 3

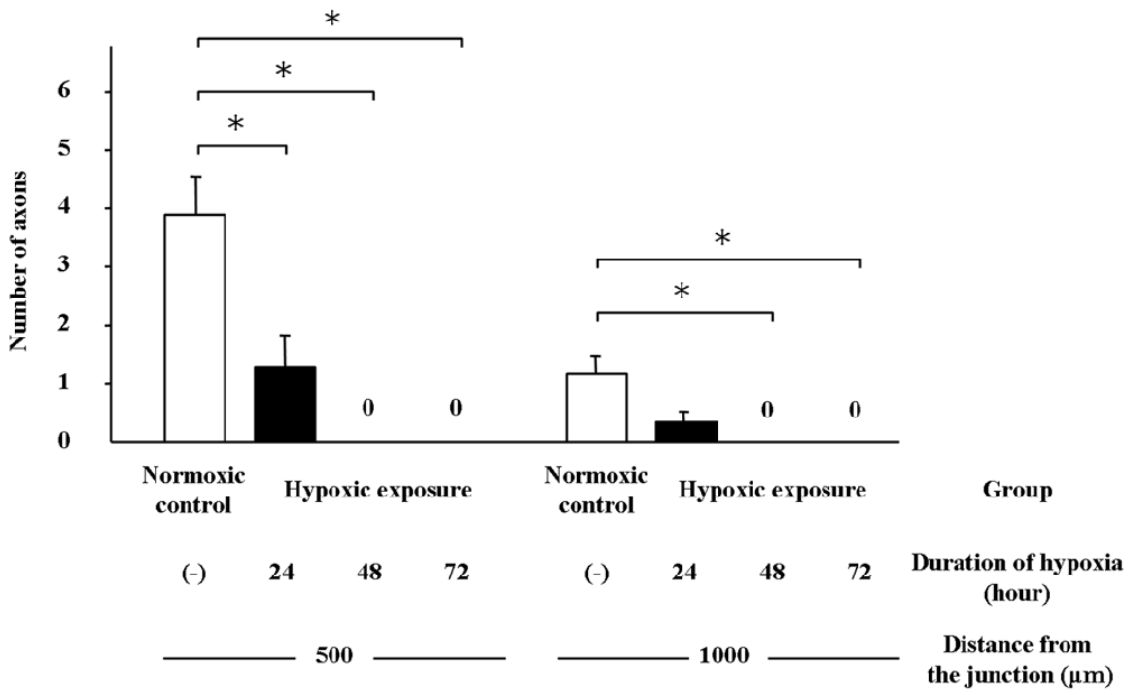

Number of axons
0
1
2
3
4
5
6
*
*
*
*
*
0
0
0
0
Normoxic control
Hypoxic exposure
Normoxic control
Hypoxic exposure
Group
(-)
24
48
72
(-)
24
48
72
Duration of hypoxia (hour)
500
1000
Distance from the junction (μm)

Fig 4

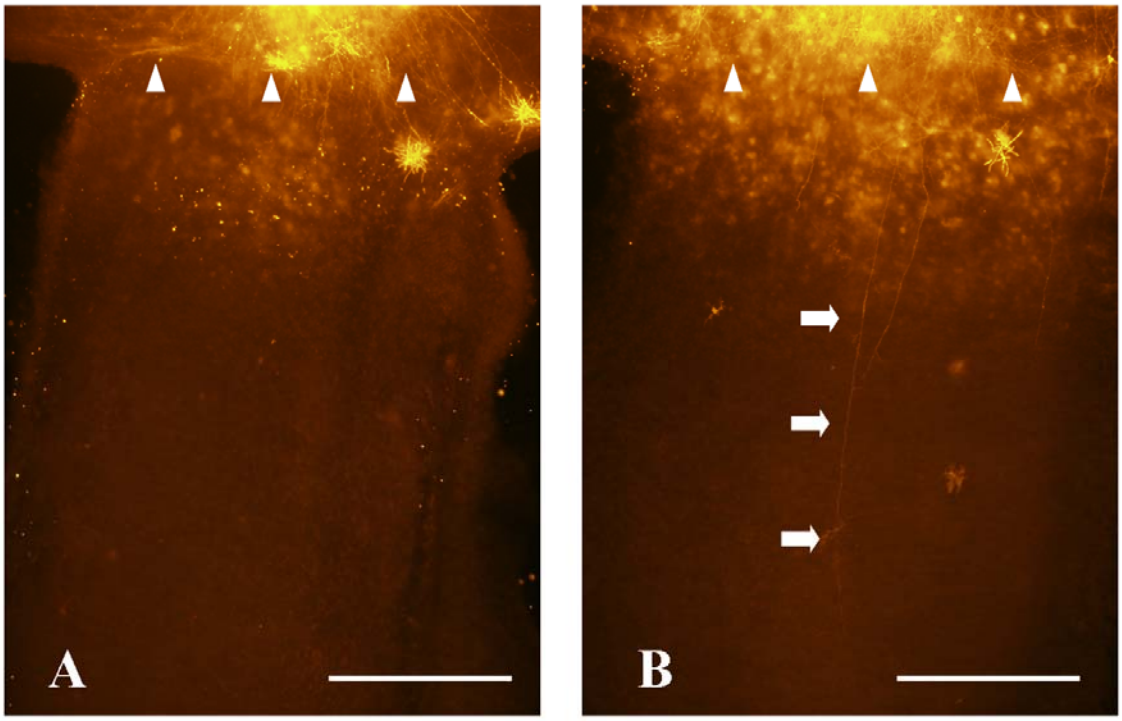

A
B

Fig 5

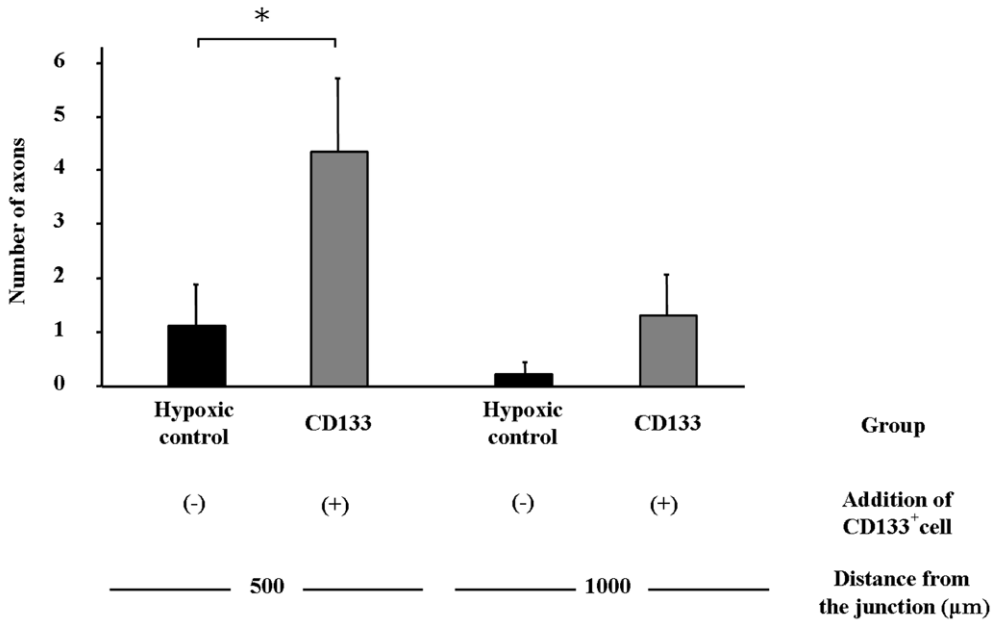

*
Number of axons
0
1
2
3
4
5
6
Hypoxic control
CD133
Hypoxic control
CD133
Group
(-)
(+)
(-)
(+)
Addition of CD133+cell
500
1000
Distance from the junction (μm)

Fig 6

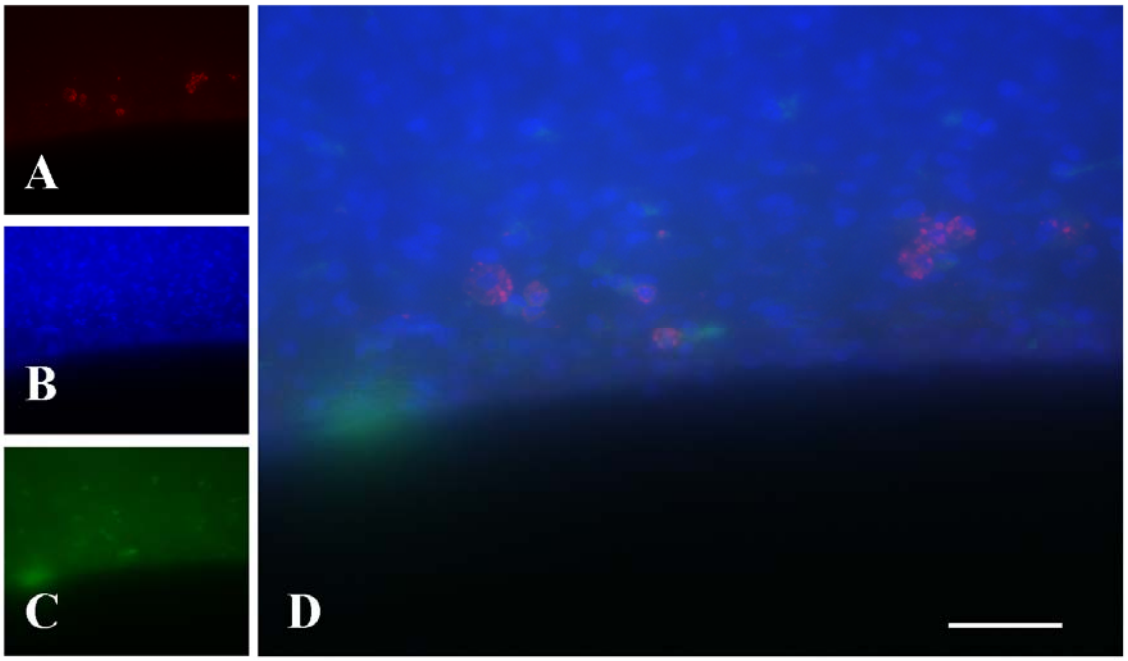

A
B
C
D

Fig 7

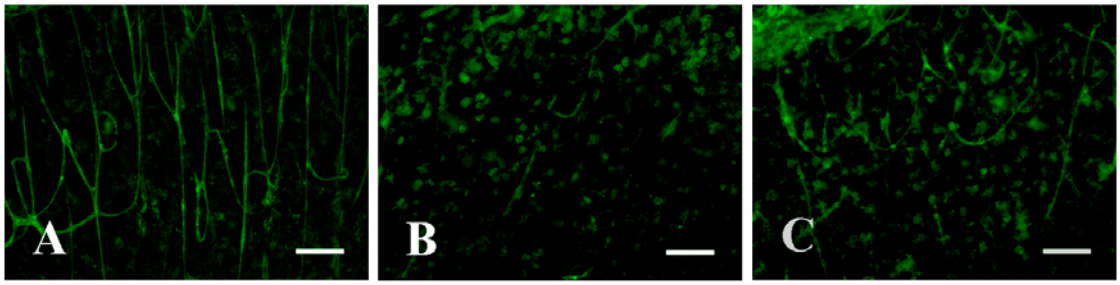

A
B
C

Fig 8

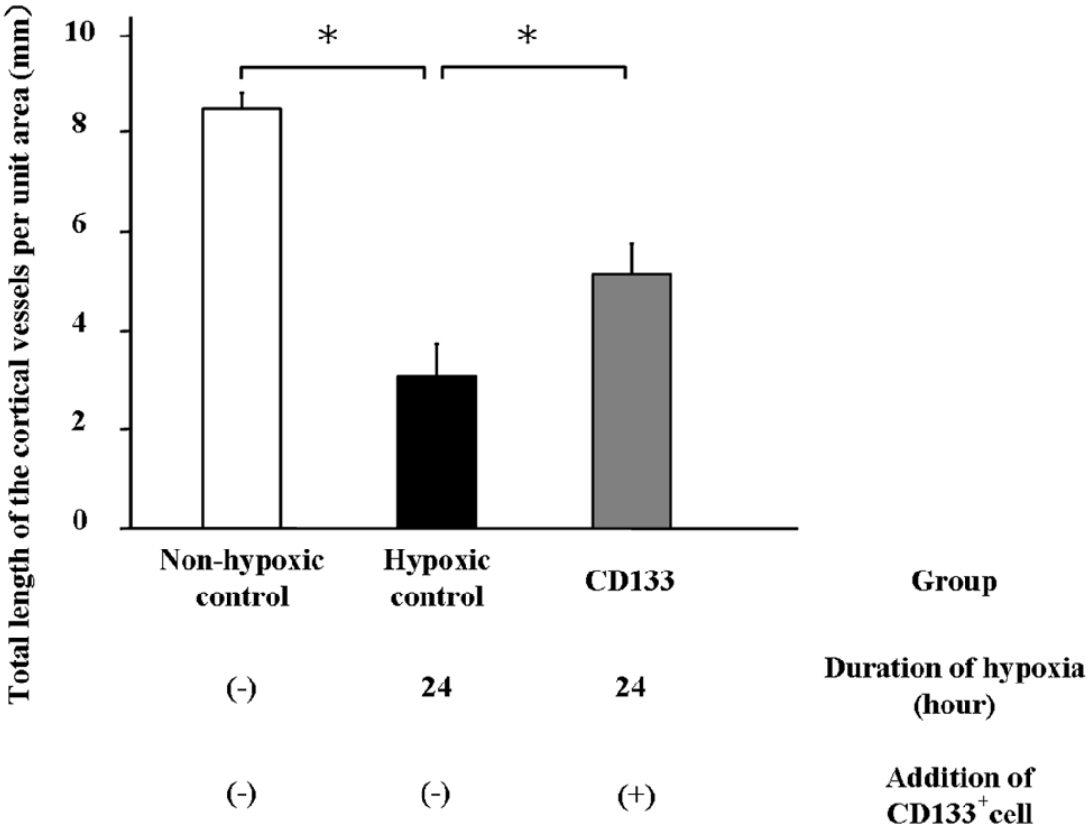

Total length of the cortical vessels per unit area (mm)
10
8
6
4
2
0
*
*
Non-hypoxic control
Hypoxic control
CD133
Group
(-)
24
24
Duration of hypoxia (hour)
(-)
(-)
(+)
Addition of CD133+cell

Fig 9

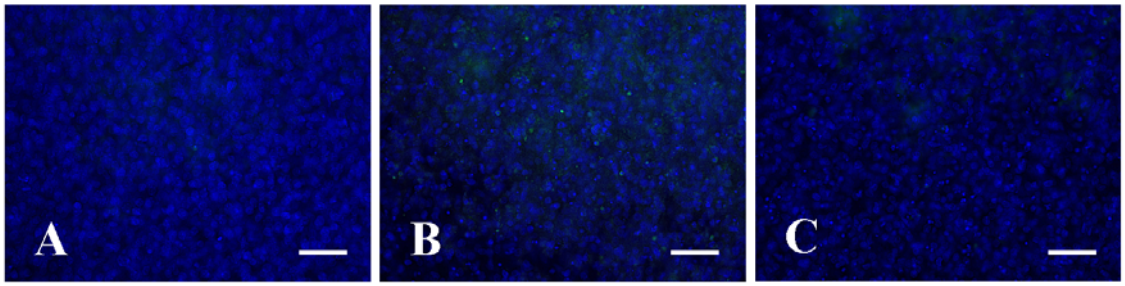
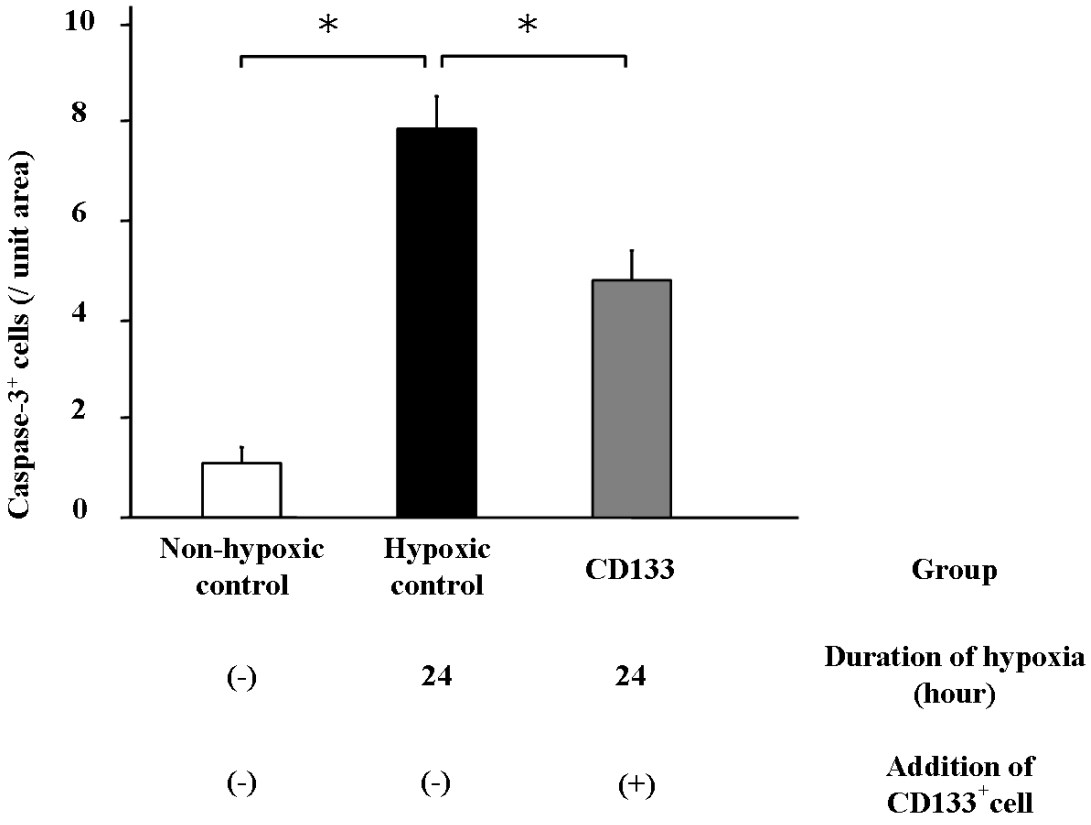

A
B
C

Fig 10


10
8
6
4
2
0
Caspase-3+ cells (/ unit area)
*
*
Non-hypoxic control
Hypoxic control
CD133
Group
(-)
24
24
Duration of hypoxia (hour)
(-)
(-)
(+)
Addition of CD133+ cell