Exercise increases neural stem cell proliferation surrounding the area of damage following rat traumatic brain injury

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Abstract Exercise enhances neuronal stem cell (NSC) proliferation and neurogenesis. However, the effect of exercise on NSC proliferation surrounding the area of damage after traumatic brain injury (TBI) is unknown. Here, we investigate the effect of running on NSC proliferation following TBI in the rat. Wistar rats received TBI and were randomly divided into two groups: (1) non-exercise group and (2) exercise group. The exercise group ran on a treadmill for 30 min/day at 22 m/min for 7 consecutive days. Immunohistochemistry was used to monitor NSC proliferation around the damaged area, and ex vivo techniques were used to isolate NSCs from the damaged region in both groups. The number of nestin- and Ki67-positive cells observed at 3 and 7 days after TBI was significantly greater in the exercise group than in the non-exercise group ($P < 0.01$). Furthermore, most nestin-positive cells in the exercise group co-localized with Ki67-positive cells. In ex vivo studies, spheres could be isolated from injured brain tissue from the exercise group at 3 and 7 days following TBI, but at only 3 days in the non-exercise group. The number of spheres isolated from injured brain tissue was greater in the exercise group than in the non-exercise group. Spheres were immunopositive for nestin and comprised NSCs that could differentiate into neurons and glia. Exercise increases the proliferation of NSCs around the damaged area following TBI. Therefore, exercise therapy (rehabilitation) in the early phase following TBI is important for recuperation from cerebral dysfunction induced by TBI.

Keywords Exercise · Neural stem cell · Traumatic brain injury · Nestin · Neurosphere

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Introduction

Traumatic brain injury (TBI) occurs as a result of a mechanical insult to the brain, which induces degeneration and death in the central nervous system (Chirumamilla et al. 2002; Rice et al. 2003). Following the initial mechanical insult, secondary pathways are activated that contribute to ischemic damage induced by circulatory disturbance, blood–brain barrier disruption and excitotoxic damage (Kawamata et al. 1995; Azbill et al. 1997; Xiong et al. 1997). These results suggest that central nervous disorders can be caused by neuronal and axonal degeneration induced by TBI (Chirumamilla et al. 2002; Rice et al. 2003). Initially, recovery from these injuries was severely limited because the neuronal loss and degeneration in the adult brain was irreversible in the mammalian nervous system.

However, recent studies have indicated that the mammalian nervous system has the potential to replenish the population of damaged and/or destroyed neurons by proliferation of neural stem cells (NSCs) (McKay 1997; Gage 2000). NSCs have been identified in adult mammals and have the potential to differentiate into either glial or neural phenotypes (Kuhn et al. 1996). Proliferation of NSCs was confirmed at two locations in the adult rodent brain: one is the subependymal zone or subventricular zone (SVZ) of the lateral ventricles (Lois and Alvarez-Buylla 1994), and the other location is the subgranular zone (SGZ) at the dentate gyrus (DG)–hilus interface (Kuhn et al. 1996). Thus, a constant slow rate of neurogenesis occurs in these areas of the adult brain (Lois and Alvarez-Buylla 1994; Kuhn et al. 1996; Parent et al. 1997).

In a recent study, treadmill running exercise was shown to increase cerebrovascular activity and physiological bioactivity in the brain (Radak et al. 2001; Wu et al. 2008; Yi et al. 2009). Moreover, treadmill running exercise increases NSC proliferation and neurogenesis in the SGZ and DG (Radak et al. 2001; Wu et al. 2008; Yi et al. 2009). In addition, treadmill running exercise has been shown to enhance the proliferation and differentiation of NSCs, enhance neurite growth and survival of neurons (Wu et al. 2008), and improve learning and memory (Wu et al. 2007). In the infarction and ischemic rat model, treadmill running exercise increases NSC proliferation in the hippocampus and SVZ, and induces and enhances neurogenesis in the brain (Komitova et al. 2005; Yagita et al. 2006; Leasure and Grider 2010).

In a recent study, we reported that NSCs could be isolated from the damaged brain region at an early stage following TBI in the rat (Itoh et al. 2005). Moreover, we identified that NSCs around the damaged area contribute to neurogenesis following TBI (Itoh et al. 2007, 2009a).

The effects of treadmill running exercise on NSCs in the hippocampus and SVZ have been investigated; however, the effects of treadmill running exercise on NSCs surrounding the damaged area following TBI remain unknown. In this study, we used the rat TBI model to investigate the effects of treadmill running exercise on NSCs surrounding damaged tissue. We hypothesize that treadmill running exercise will enhance NSC proliferation in the region surrounding the damaged area following TBI.

Materials and methods

Animals and surgical procedures

Adult male Wistar rats (10 weeks of age, weighing 200–250 g, $n = 72$) were housed at 22°C under a 12:12-h light dark cycle and had access to food and water ad libitum. All experiments were carried out with the approval of the Institutional Animal Experimentation Committee of Kinki University School of Medicine. Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The scalp was incised on the midline and the skull was exposed. A 2-mm hole was drilled (1 mm posterior, +1 mm right lateral to the bregma) in the right parietal calvaria (Itoh et al. 2005, 2007). Brain injury above the dura mater was inflicted with a pneumatic controlled injury device (Itoh et al. 2005, 2007) at an impact velocity of 4 m/s, with an impact tip diameter of 1 mm and a fixed impact deformation depth of 2 mm from the cerebral surface.

Following TBI, rats were randomly divided into two groups: (1) non-treadmill running exercise group (non-exercise group, $n = 36$) and (2) treadmill running exercise group (exercise group, $n = 36$). The running protocol was performed according to that of Uda et al. (2006). To minimize novelty stress, all rats were allowed to become familiarized with the treadmill by running on a motorized treadmill (Brain Science Idea Corporation, Osaka, Japan) for 15 min/day at 15 m/min for 7 consecutive days. Electrical stimulation to encourage rats to run was disconnected to avoid pain stress after familiarization. The exercise group ran for 30 min/day at 22 m/min for 7 consecutive days. The non-exercise group rats were put on the treadmill for 30 min/day for 7 consecutive days without running.

Immunohistochemistry

At 1, 3 and 7 days following TBI and treadmill running, seven rats in each of the non-exercise and exercise groups were deeply anesthetized by an intraperitoneal injection of pentobarbital (150 mg/kg) and then subjected to sequential intracardial perfusion with 300 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.4–7.5), followed by 300 ml of 4% (w/v) paraformaldehyde (PFA) in PBS. Brains were removed and stored in PFA for 3 days, before the lesion was sliced into serial coronal sections (20 μm thick) using a microslicer (Dousaka EM, Kyoto, Japan). Each section was treated with 3% (v/v) H_2O_2 in Tris-buffered saline (TBS; 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) for 30 min to block endogenous peroxidase activity. Sections were washed three times with TBS containing 0.1% (v/v) Triton X-100 (TBS-T), blocked with 3% (v/v) bovine serum albumin (BSA; Sigma, St. Louis, MO) in TBS-T for 30 min and incubated overnight at room temperature with the following primary antibodies in blocking solution: (1) monoclonal antibody against the rat NSC marker, nestin (1:10000, BD Biosciences Pharmingen, San Diego, CA) and (2) mouse monoclonal antibody against the cell proliferation marker, Ki67 (1:10000, BD Biosciences Pharmingen, San Diego, CA). Following extensive washing,

sections were further incubated with a HISTIFINE Rat-PO (mouse)-kit, comprising peroxidase-conjugated anti-mouse secondary antibody, for 60 min at room temperature. The HISTIFINE Rat-PO kit contained pre-absorbed rat serum and exhibited negligible non-specific binding of rat serum in injured rat tissues. Labeling was visualized using diaminobenzidine (DAB; Vector Peroxidase Substrate Kit; Vector Laboratories, Burlingame, CA) for 5 min, and the sections were counterstained with hematoxylin to quantify the number of nestin-positive cells. Negative control staining was performed with normal mouse serum instead of primary antibodies, following the procedure outlined above.

Quantification of nestin- and Ki67-positive cells

All DAB-labeled cells within 500 μm of the edge of the damaged region (cortex), excluding white matter, following TBI, were counted in three serial sections under a Nikon E 1000 M microscope (Nikon Corporation, Tokyo, Japan) using a 20× objective. The number of DAB-labeled cells in three serial sections was averaged. An image of the measured area was captured at 20× magnification using a CCD camera (ACT-2U; Nikon Corporation), and the measured area and volume in each image were traced and measured by computer. The number of DAB-labeled cells was calculated from the average DAB-labeled positive number and the volume. The number of nestin- and Ki67-positive cells were expressed as the number of positive cells/100 μm^3 (Itoh et al. 2010a, b).

Double-immunofluorescence staining
for nestin and Ki67

At 3 and 7 days post-TBI, sections were washed and blocked with 50 mM glycine in TBS for 2 h at 37°C to reduce non-specific fluorescence. Sections were washed with TBS-T, blocked with 3% (v/v) BSA in TBS-T and incubated with a monoclonal anti-nestin antibody (1:300 dilution; BD Biosciences Pharmingen) overnight at room temperature. Following extensive washing, sections were further incubated with Alexa Fluor 488 anti-rabbit IgG (1:300 dilution; Molecular Probes, Inc., Eugene, OR, USA) for 80 min at room temperature. Sections were washed and incubated with a rabbit polyclonal anti-Ki67 antibody (1:300 dilution; Millipore, Billerica, MA, USA) overnight at room temperature. Following extensive washing, sections were further incubated with Alexa Fluor 555 anti-rabbit IgG (1:300 dilution; Molecular Probes, Inc., Eugene, OR, USA) for 80 min at room temperature. Sections were viewed using a confocal laser-scanning microscope (LSM5 PASCAL; Carl Zeiss Jena GmbH, Jena, Germany).

Isolation and culture of NSCs

NSCs were isolated and cultured according to the method described previously (Itoh et al. 2005). At 1, 3 and 7 days following TBI in the non-exercise and exercise groups, cerebral cortex tissue 2 mm in diameter, measured from the center of the lesion, was separated by gross dissection under a dissecting microscope (Itoh et al. 2005). Care was taken to remove and discard the meninges and blood vessels. One cerebral tissue sample from five individual rats from each of the two experimental groups was taken for NSC culture. The tissue was cut into small pieces and dissociated by incubation in Hanks' Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA) containing 0.1% (w/v) trypsin (Invitrogen) and 0.01% (w/v) DNase 1 (Roche, Indianapolis, IN, USA) at 37°C for 30 min. An equal volume of fetal calf serum (Invitrogen) was added to the tissue suspension and centrifuged at 100×g for 5 min. The supernatant was removed, HBSS was added, and cells were dissociated by trituration and then centrifuged at 100×g for 5 min. The cells were plated as a single-cell suspension on ornithine- and fibronectin-coated 60 mm culture dishes in plating medium (N2/DF) consisting of Dulbecco's modified Eagle's medium (Invitrogen)/F-12 medium (Invitrogen) supplemented with basic fibroblast growth factor (bFGF, 20 ng/ml, Roche, Indianapolis, IN, USA), epidermal growth factor (EGF, 20 ng/ml, Roche), insulin (25 μg/ml, Roche), transferrin (100 μg/ml, Roche) and progesterone (100 μg/ml, Wako, Osaka, Japan), and maintained at 37°C in a humidified 5% CO_2 atmosphere for 3 days. After 4 days, cells that had attached to the bottom of the culture dish were desquamated by trituration, and collected and dissociated by trituration in N2/DF medium. The single-cell suspension was replated on untreated culture dishes, and after 10–14 days in culture spheres had formed. After NSC culture, the number of spheres per culture dish was counted ($n = 5$) and sphere differentiation was investigated by culturing without bFGF and EGF on ornithine-coated round cover glasses (1 cm^2) in 24-well culture plates at 37°C in a humidified 5% CO_2 atmosphere for 4 days.

Immunostaining of cultured cells and correlation
of Ki67-positive cell number with isolated sphere
number

Immunostaining was performed on spheres and 4-day cultures that had been fixed using 4% (w/v) PFA in PBS for 20 min. Spheres were stained with anti-rat nestin monoclonal antibody and either the neuronal marker Tuj1 antibody (1:300 dilution, Roche), the astrocyte marker glial fibrillary acidic protein (GFAP) antibody (1:300 dilution, DAKO) or the oligodendrocytes marker O4
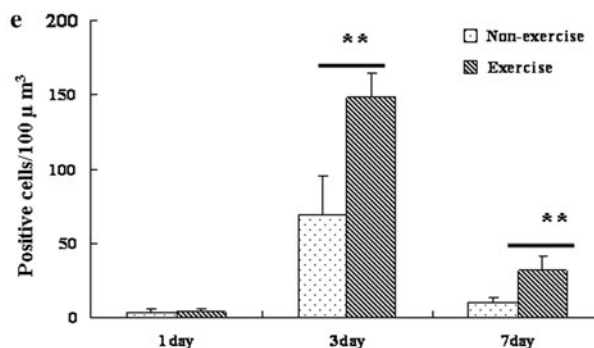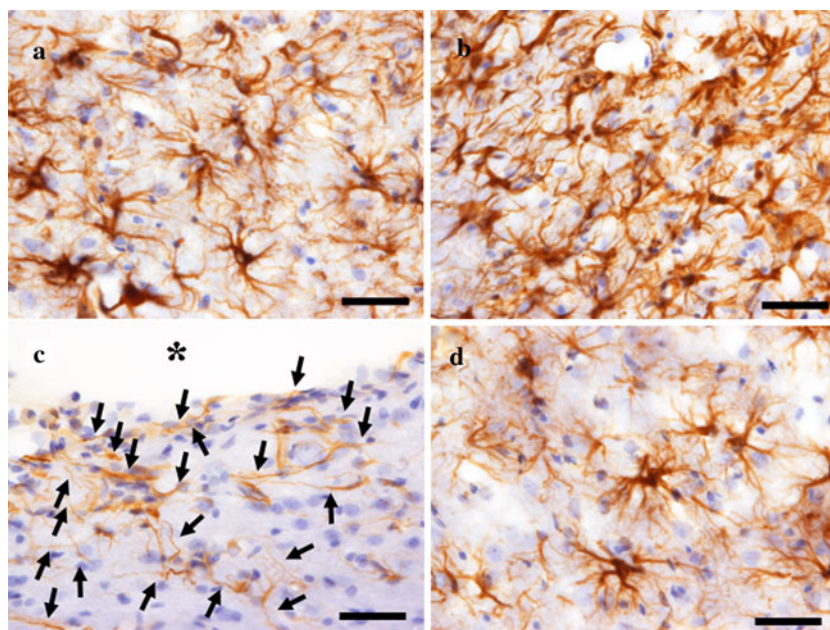
antibody (1:300 dilution, Roche). The secondary antibodies used were Alexa Fluor 488 anti-mouse IgG antibody (1:300 dilution, Molecular Probes, Inc.) for Tuj1, Alexa Fluor 555 anti-rabbit IgG antibody (1:300 dilution, Molecular Probes, Inc.) for GFAP and Alexa Fluor 488 anti-mouse IgG antibody (1:300 dilution, Molecular Probes, Inc.) for O4. All antibodies were diluted in TBS-T containing 3% (v/v) BSA. The cells cultured for 4 days were used for fluorescence double labeling with Tuj1 and GFAP.

Correlation of Ki67-positive cell number and isolated sphere number in the no-exercise and exercise groups was determined.

Statistical analysis

Data were expressed as a mean $\pm$ SD. Statistical analysis was performed using one-way analysis of variance with Fisher's post hoc test (Stat View$^{\circledR}$; SAS Institute Inc, Cary, NC, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Quantification of nestin-immunopositive cells following TBI

At 1 day following TBI in the non-exercise and exercise groups, a few small nestin-positive cells were present around the damaged area. At 3 days following TBI in the non-exercise and exercise groups, there were many larger nestin-positive cells around the damaged area, and these cells possessed nestin-immunopositive cytoplasm and projections morphologically similar to those of small reactive astrocytes (Fig. 1a, b). Furthermore, there was an increase in the number of larger cells in the exercise group than in the non-exercise group (Fig. 1a, b). At 7 days following TBI in the non-exercise group, there were a few nestin-positive fibers among the cells around the damaged area (Fig. 1c). However, in the exercise group, there were many larger sized nestin-positive cells, which possessed a nestin-immunopositive cytoplasm and projections similar to reactive astrocytes (Fig. 1d).

Fig. 1 Immunostaining for nestin around the damaged cerebral cortex following TBI in the rat. At 3 days after injury in the non-exercise (**a**) and exercise (**b**) groups, abundant nestin immunoreactivity was present mainly in the cytoplasm and projections. At 7 days after injury in the non-exercise group, nestin-immunopositive elongating fibers were seen (**c**, *arrows*), while in the exercise group, abundant nestin immunoreactivity was present mainly in the cytoplasm and projections (**d**). * Damaged region. *Scale bar* = 50 μm. Graph showing nestin-immunopositive cell numbers around the damaged cerebral cortex following TBI in the rat (**e**). The results are shown as mean $\pm$ SD. ** $P < 0.01$; $n = 7$

The number of nestin-positive cells is shown in Fig. 1e. At 1 day following TBI, the nestin-positive cell number was 4.2 ± 2.6 and 5 ± 2.1 in the non-exercise and exercise groups, respectively. The number of nestin-positive cells in the exercise group did not show a significant increase compared with the number in the non-exercise group. However, the nestin-positive cell number at 3 and 7 days following TBI in the exercise group (148.2 ± 16.9, 31.5 ± 9.9, respectively) showed a significant increase compared with the number in the non-exercise group at 3 and 7 days (67.1 ± 26.7 and 9.8 ± 4.8, respectively, $P < 0.01$).

Quantification of Ki67-immunopositive cells following TBI

At 1 day following TBI in the non-exercise and exercise groups, a few small Ki67-positive cells were present around the damaged area. At 3 days following TBI in the non-exercise (Fig. 2a) and exercise groups (Fig. 2b), there were many Ki67-positive cells around the damaged area. Furthermore, there were more of these cells in the exercise group than in the non-exercise group (Fig. 2a, b). At 7 days following TBI in the non-exercise group, there were only a few Ki67-positive cells around the damaged area (data not shown). However, in the exercise group, there were many Ki67-positive cells (data not shown).

The number of Ki67-positive cells is shown in Fig. 2c. At 1 day following TBI, the Ki67-positive cell number was 5.2 ± 2.3 and 6.1 ± 1.9 in the non-exercise and exercise groups, respectively. The number of Ki67-positive cells in the exercise group did not show a significant increase compared with the number in the non-exercise group. However, the Ki67-positive cell number at 3 and 7 days following TBI in the exercise group (395.7 ± 118.9, 110.3 ± 58, respectively) showed a significant increase compared with the number in the non-exercise group at 3 and 7 days (221.3 ± 44.9 and 9.2 ± 7.4, respectively, $P < 0.01$).

Double-immunofluorescence staining for nestin and Ki67

There were many nestin-positive cells and Ki67-positive cells in the non-exercise and exercise groups (Fig. 3a–f) around the damaged area at 3 days after TBI. The majority of nestin-positive cells were also positive for Ki67 immunostaining in the non-exercise (Fig. 3c) and exercise (Fig. 3f) groups. There were nestin-positive projections (Fig. 3g), and these projections were not immunopositive for Ki-67 (Fig. 3h) in the non-exercise group at 7 days after TBI (Fig. 3i). However, nestin-positive cells (Fig. 3j) were reactive for Ki67 (Fig. 3k) in the exercise group at 7 days after TBI (Fig. 3l).

NSC isolation, counting and immunostaining

At 1 day following TBI in the non-exercise and exercise groups, it was not possible to isolate spheres surrounding the damaged area of the cerebral cortex (Fig. 4a).

Fig. 2 Immunostaining for Ki67 around the damaged cerebral cortex at 3 days following TBI in the rat. At 3 days after injury in the non-exercise (**a**) and exercise (**b**) groups, abundant Ki67 immunoreactivity was present mainly in the nuclei. *Scale bar* $= 50$ μm. Graph showing Ki67-immunopositive cell numbers around the damaged cerebral cortex following TBI (**c**). The results are shown as a mean $\pm$ SD. ** $P < 0.01$; $n = 7$
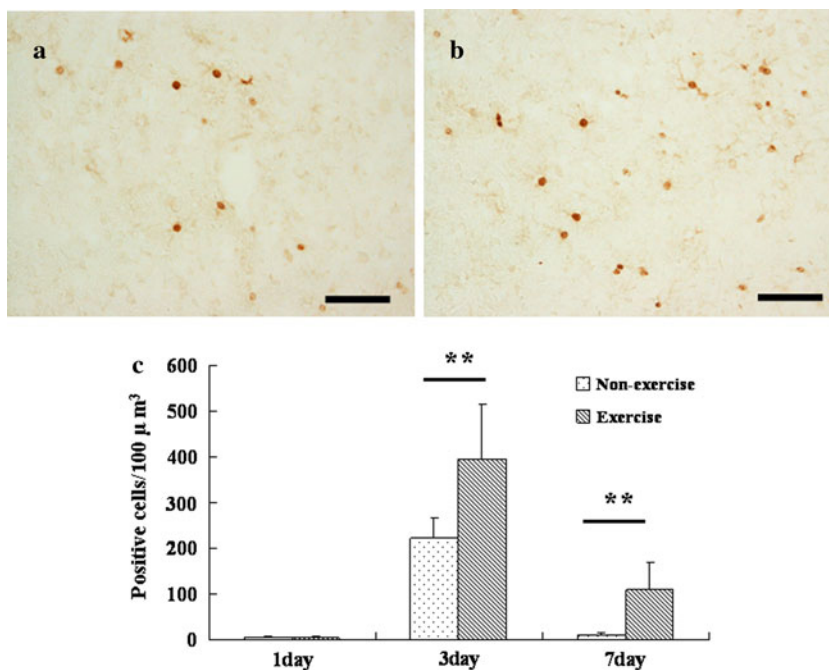
Fig. 3 Double-immunofluorescence staining of nestin and Ki67 around the damaged area at 3 and 7 days after TBI. At 3 days after the injury, many nestin-immunopositive cells were observed in the non-exercise (**a**, *green*) and exercise (**d**, *green*) groups. The Ki67-immunopositive cells (**b** and **e**, *red*) were double-immunopositive for nestin in the non-exercise (**c**, *arrows*) and exercise (**f**, *arrows*) groups. At 7 days after injury in the non-exercise group, many nestin-immunopositive projections (**g**, *green*) were observed. Double-labeling of Ki67-immunopositive cells (**h**, *red*) with nestin was not seen (**i**). Meanwhile, at 7 days after injury in the exercise group, many nestin-immunopositive cells (**j**, *green*) were observed. Double-labeling of Ki67-immunopositive cells (**k**, *red*) with nestin was seen (**l**, *arrows*). * Damaged region. *Scale bar* = 50 μm
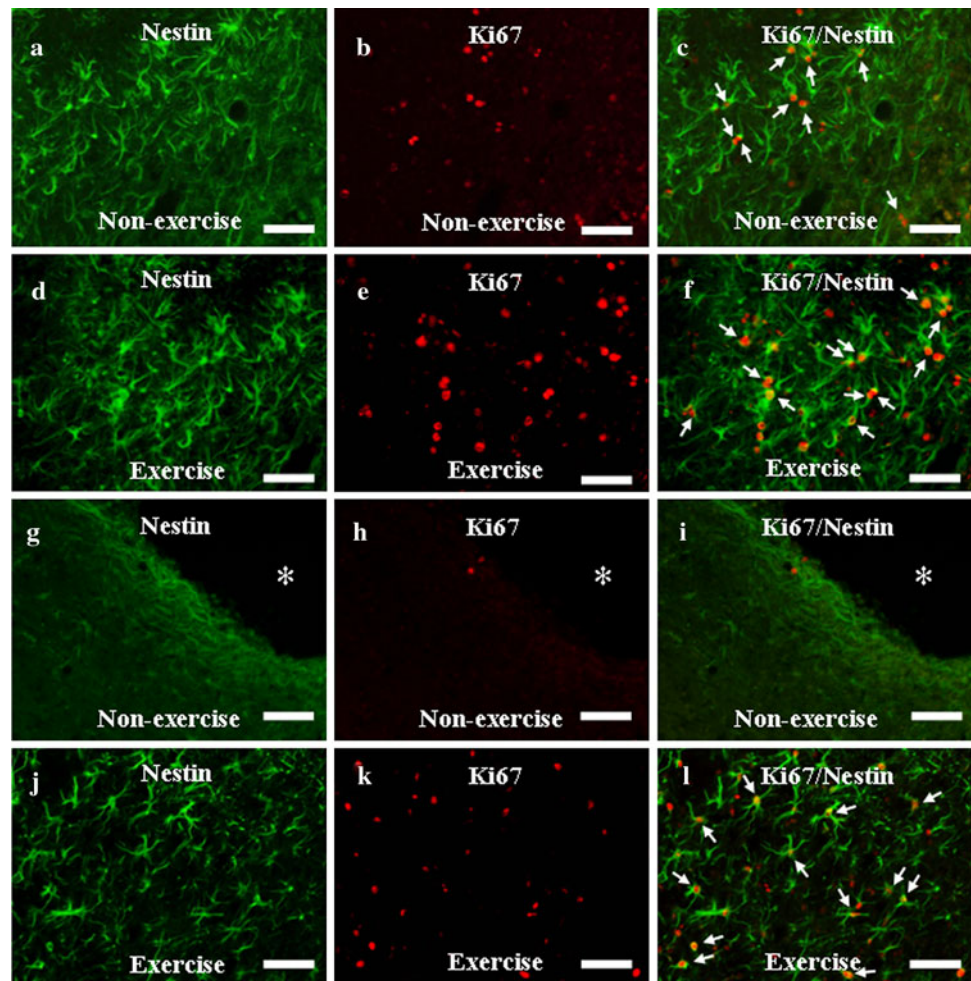
However, at 3 days after injury in the non-exercise and exercise groups, spheres were isolated and cultured (Fig. 4b). Moreover, at 7 days after injury in the exercise group, but not in the non-exercise group, spheres were isolated and cultured. Almost all aggregated cells in spheres isolated from non-exercise and exercise groups showed nestin immunoreactivity (Fig. 4c). In addition, the spheres were not immunopositive for Tuj1, a marker of immature neuronal cells, or vimentin, a marker of immature glial cells (data not shown).

At 3 and 7 days following injury, the number of isolated and cultured spheres in the exercise group (17 ± 4 and 2 ± 3, respectively) significantly increased compared with the number in the non-exercise group at 3 and 7 days (10 ± 6 and 0, respectively) (Fig. 4d, $P < 0.01$).

Differentiation of cultures

Phase-microscopic images of cultured and differentiated cells isolated from the exercise group after 4 days in culture are shown in Fig. 5a. The spheres isolated from the non-exercise and exercise groups immediately attached to the bottom of the cell culture dishes. The number of fibers that elongated from the spheres increased with time. After 4 days in culture, many cells with elongated fibers had migrated from the spheres (Fig. 5a). Cells labeled positive for O4 in the cytoplasm (Fig. 5b), and Tuj1-immunopositive labeling was also found in the cytoplasm and elongated fibers (Fig. 5c). In addition, there were also cells with GFAP-immunopositive labeling in the cytoplasm (Fig. 5d). However, Tuji1-immunopositive labeling did not co-localize with GFAP-immunopositive labeling (Fig. 5e).

Correlation of Ki67-positive cell number with isolated sphere number

Correlation of Ki67-positive cell number with isolated sphere number is shown in Fig. 6. The number of Ki67-positive cells in both the non-exercise and exercise groups significantly correlated with the number of spheres isolated from the damaged brain tissue after TBI (Fig. 6, $y = 0.0527x - 0.6928$, $R^2 = 0.9901$, $n = 7$, $P < 0.01$).

Fig. 4 Photomicrographs of isolated spheres from the area surrounding the injury in the exercise group. No spheres were observed in the cultures derived from 1-day tissue in the exercise group (**a**). In the exercise group, a few spheres were found in the cultures derived from the 3-day tissue after 13 days of culture (**b**). Isolated spheres showing nestin immunoreactivity (**c**). *Scale bar* = 50 μm. Graph showing the number of isolated and cultured spheres from the area surrounding the injury (**d**). The results are shown as a mean ± SD. ** $P < 0.01$; $n = 5$
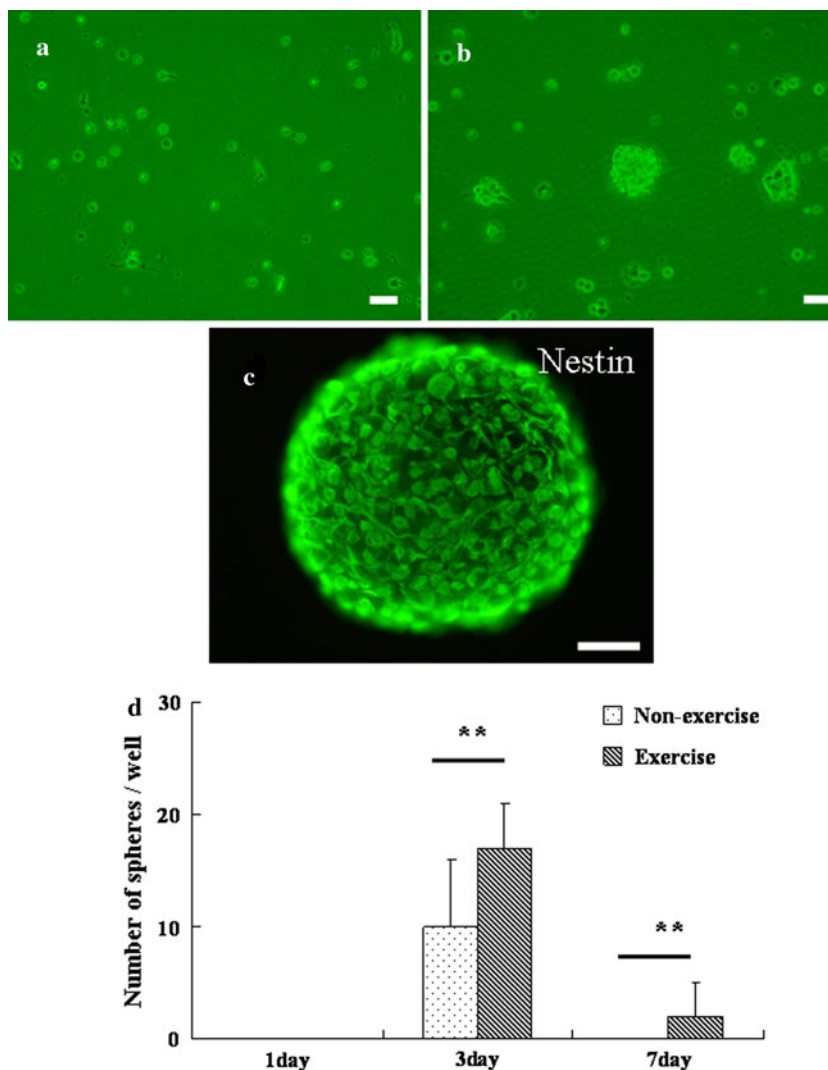
Discussion

In this study, nestin-positive cells were present 1–7 days after TBI in the non-exercise and exercise groups. In both groups, the nestin-positive immunoreactive cells reached their maximum number at 3 days following TBI. This result is consistent with our previous reports (Itoh et al. 2005, 2007) and other studies where the number of nestin-positive cells increased around the damaged cerebral cortex at 1–4 days after cryo-injury (Moon et al. 2004) and reached a maximum 3 days after ablation injury (Douen et al. 2004). Nestin-positive cells were present around the damaged cerebral cortex at 1–7 days following controlled cortical impact (CCI), and the number of nestin-positive cells reached a maximum at 4 days after CCI (Chen et al. 2003). Our results, and others, indicate that the maximal numbers of nestin-positive cells are present around the damaged area at 3 or 4 days after injury.

The number of Ki67-positive cells at the early stage following TBI in the exercise group showed a significant increase compared with the non-exercise group. Moreover, many of the nestin-positive cells showed Ki67 immunoreactivity in the exercise group. The mitogenic factor, bFGF, enhances and accelerates proliferation and cell division of NSCs (Itoh et al. 2006, 2007). Exercise increases the production of bFGF in the hippocampus and SVZ in the rat brain. Given that bFGF accelerates cell division of nestin-positive NSCs and induces neurogenesis in the rat brain (Wu et al. 2007), it is possible that exercise induces neurogenesis in the rat brain (Wu et al. 2007). In addition, exercise increases the production of brain-derived neurotrophic factor and increases trkB receptors on NSCs in the hippocampus and SVZ (Wu et al. 2008). In these studies, cell division and proliferation of NSCs was enhanced and neurogenesis was accelerated (Wu et al. 2008).

Fig. 5 Phase-contrast and immunohistochemical images of cells differentiated from neurospheres without bFGF and EGF after 4 days of culture in the running group. Phase-contrast microscopic images showing cells differentiated from neurospheres in culture. *Arrow* indicates neurospheres (**a**). Differentiated cultures of neurospheres with Alexa Fluor 488 immunostaining showing O4-immunoreactive cells (*green*, **b**). Double-labeling fluorescence immunostaining showing Tuj1-immunoreactive cells (*green*, **c**) and GFAP-immunoreactive cells (*red*, **d**). A merged image of **c** and **d** is shown **e**. Tuj1 and GFAP do not co-localize. *Scale bar* = 50 μm
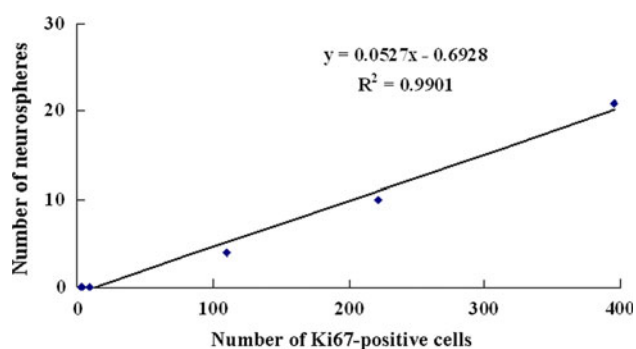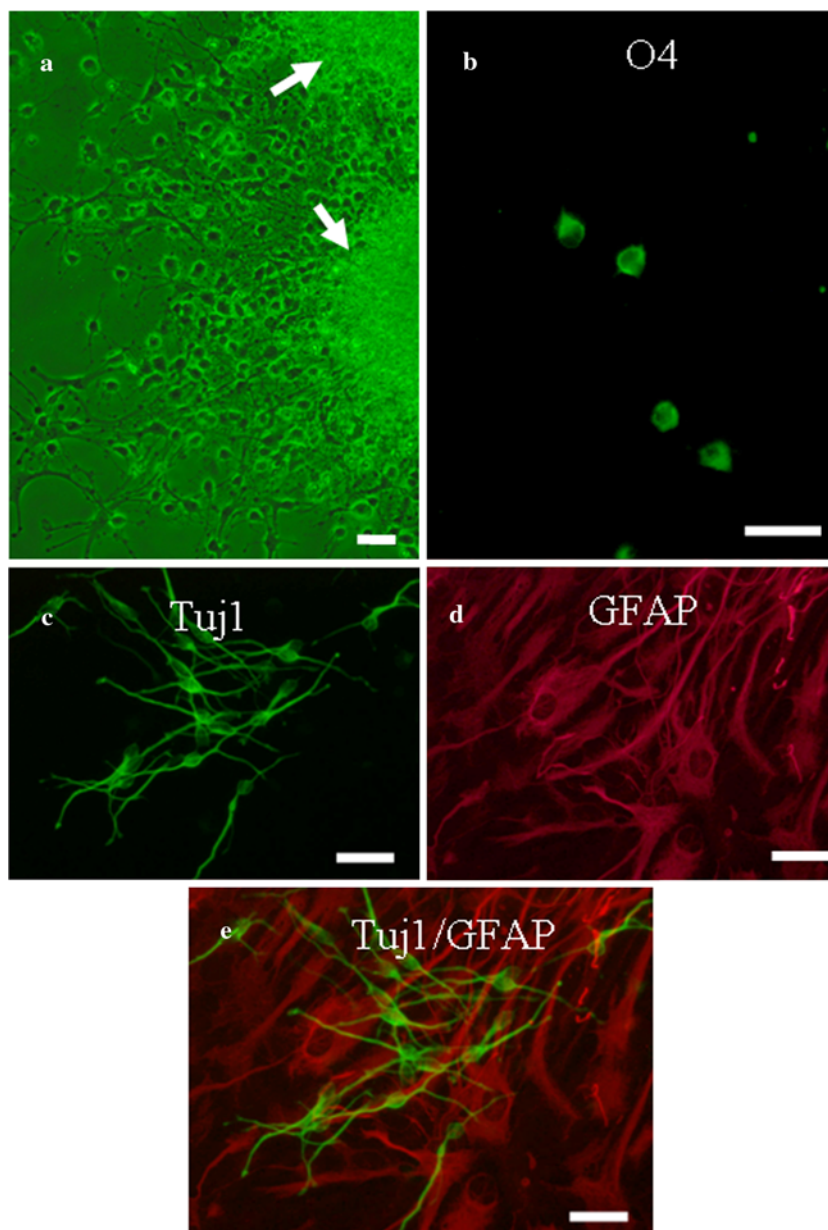

Fig. 6 Correlation of Ki67-positive cell number with the number of isolated neurospheres around the damaged area following rat TBI

Furthermore, exercise increases the production of insulin-like growth factor 1 (Llorens-Martin et al. 2010) and growth hormone (Blackmore et al. 2009) in the brain. These growth factors accelerate NSC division and proliferation, and increase and enhance neurogenesis in the hippocampus and SVZ (Blackmore et al. 2009; Llorens-Martin et al. 2010). In the rat ischemic model, exercise after brain injury has been shown to increase the ability of NSCs to divide and proliferate, and neurogenesis is enhanced by exercise in the hippocampus and SVZ (Komitova et al. 2005; Luo et al. 2007). From these results, it appears that nestin- and Ki67-positive cell numbers in the exercise group increase compared with the non-exercise

group in the early phase following TBI and, moreover, nestin-positive cells with Ki67 immunoreactivity in the exercise group increase compared with the non-exercise group. These results indicate that in the early phase following TBI, many more NSCs have the ability to divide in the exercise group than in the non-exercise group.

In an ischemic rat model, exercise has been shown to reduce oxidative stress induced by hydroxyl radicals ($\cdot OH$) and superoxide (O_2^-) after ischemia (Radak et al. 2001). Recently, we reported that $\cdot OH$ and O_2^- after TBI induced neuronal and NSC death (Itoh et al. 2009b, 2010a); however, radical scavengers absorbed $\cdot OH$ and O_2^- induced by TBI and subsequently inhibited neuronal and NSC death (Itoh et al. 2009b, 2010a). These results suggest that exercise may alleviate NSC damage and, therefore, increase nestin-positive numbers because exercise decreases oxidative stress after TBI.

At 1, 3 and 7 days following TBI in the non-exercise and exercise groups, the cerebral cortex (without white matter) was dissected from the region surrounding the damaged area for the isolation of nestin-positive cells. At 3 days following TBI in the non-exercise and exercise groups, nestin-positive spheres could be isolated from the surrounding damaged brain tissue, but the number of spheres in the exercise group increased significantly compared with the non-exercise group. Furthermore, at 7 days following TBI, nestin-positive spheres could be isolated from the region surrounding the damaged brain tissue in the exercise group, but not the non-exercise group. In Fig. 6, the number of Ki-67-positive cells around the damaged area following TBI correlated with the number of spheres isolated from brain tissue. Therefore, these data may indicate that the number of Ki67-positive cells around the damaged area correlates with the number of NSCs present.

In this study, the number of nestin- (NSCs) and Ki67-positive cells at 3 and 7 days following TBI in the exercise group showed a significant increase compared with the non-exercise group around the damaged area. Therefore, it appears that exercise enhances the proliferation of nestin-positive cells, including NSCs, in the region surrounding the damaged area after TBI. These results indicate that the presence of both nestin- and Ki67-postive cells around the damaged area is important in the early phases following TBI for possible regeneration of damaged tissue.

In our immunohistochemical studies, almost all aggregated cells in the spheres isolated from the non-exercise and exercise groups showed nestin immunoreactivity, but not Tuj1 or vimentin immunoreactivity. Neurospheres comprised NSCs that had the potential to differentiate into neurons or glia following removal of bFGF or EGF, which are mitogenic factors in the culture medium (Itoh et al. 2005). Neurospheres originating from the rat brain differentiate into Tuj1-positive neurons and GFAP-positive astrocytes after 2–4 days of culture without bFGF in the culture medium (Yamamoto et al. 2001). In this study, spheres differentiated into Tuj1-, GFAP- and O4-positive cells after 4 days of culture without bFGF in the culture medium. Our results confirm our recently reported findings (Itoh et al. 2005) that neurospheres isolated from the damaged region at an early stage following rat TBI are NSCs that have the ability to differentiate into neurons and glia.

Recently, it has been reported that NSCs migrate to injured regions from the SVZ or SGZ of the DG–hilus after brain injury (Lois and Alvarez-Buylla 1994; Kuhn et al. 1996; Parent et al. 1997; Tani et al. 2010). In contrast, reactive astrocytes that may be identified by GFAP immunoreactivity appear around the damaged area after brain injury (Seri et al. 2001; Picard-Riera et al. 2004). In this study, there were many GFAP-positive reactive astrocytes surrounding the damaged area after TBI. Reactive astrocytes around the damaged area undergo blastogenesis following brain injury to give rise to NSCs that are immunopositive for nestin and can differentiate into neurons and glia (Seri et al. 2001; Picard-Riera et al. 2004). In the SVZ or the SGZ, reactive astrocytes undergo blastogenesis following brain injury and differentiate into neural precursor cells that are immunopositive for both nestin and GFAP (Seri et al. 2001; Picard-Riera et al. 2004). Also, we reported that cultured rat type I astrocytes undergo blastogenesis when cultured in the presence of bFGF to become nestin-positive NSCs, which can differentiate into neuronal and glial cells (Itoh et al. 2006). Therefore, the occurrence of reactive astrocytes around the damaged area following brain injury appears to be important. However, it is unclear whether NSCs migrate to the damaged area from the SVZ and SGZ, or whether astrocytes undergo blastogenesis following injury and differentiate into nestin-positive cells.

From our current study, we propose that exercise in the early phase following TBI increases the number of nestin-positive cells, including NSCs, which have the potential to differentiate into neurons and glia, and enhances proliferation of NSCs around the damaged area. Therefore, exercise therapy (rehabilitation) in the early phase following TBI may be important for recuperation of induced cerebral dysfunction following TBI.

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