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INTACT AND INJURED ENDOTHELIAL CELLS DIFFERENTIALLY MODULATE POSTNATAL MURINE FOREBRAIN NEURAL STEM CELLS

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Abstract

Neural stem cells (NSCs) persist in the forebrain subventricular zone (SVZ) within a niche containing endothelial cells. Evidence suggests that endothelial cells stimulate NSC expansion and neurogenesis. Experimental stroke increases neurogenesis and angiogenesis, but how endothelial cells influence stroke-induced neurogenesis is unknown. We hypothesized intact or oxygen-glucose deprived (OGD) endothelial cells secrete factors that enhance neurogenesis. We co-cultured mouse SVZ neurospheres (NS) with endothelial cells, or differentiated NS in endothelial cell-conditioned medium (ECCM). NS also were expanded in ECCM from OGD-exposed (OGD-ECCM) endothelial cells to assess injury effects. ECCM significantly increased NS production. NS co-cultured with endothelial cells or ECCM generated more immature-appearing neurons and oligodendrocytes, and astrocytes with radial glial-like/reactive morphology than controls. OGD-ECCM stimulated neuroblast migration and yielded neurons with longer processes and more branching. These data indicate that intact and injured endothelial cells exert differing effects on NSCs, and suggest targets for stimulating regeneration after brain insults.

Keywords

Endothelial Cells; Neural stem cell; Neurogenesis; Oxygen-glucose deprivation; Stroke; Subventricular Zone

INTRODUCTION

Neural stem cells (NSCs) persist in the mammalian forebrain subventricular zone (SVZ) and generate olfactory bulb interneurons (Altman, 1969; Kaplan and Hinds, 1977). Factors that maintain SVZ NSCs in a quiescent state or stimulate them after injury are largely unknown,

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but some cues likely derive from cells in the local environment, as SVZ NSCs reside within a niche containing vascular, glial and ependymal elements. Endothelial cells prominently influence the neurogenic niches of adult rodent and songbird (Leventhal et al., 1999; Louissaint et al., 2002; Palmer et al., 2000). Manipulating angiogenesis influences neurogenesis in the songbird higher vocal center (Louissaint et al., 2002), and rodent SVZ explants co-cultured with endothelial cells generate more neurons (Leventhal et al., 1999). SVZ-derived neuroblasts also migrate alongside blood vessels to reach the olfactory bulb (Bovetti et al., 2007). Non-contact co-cultures of endothelial cells with embryonic cortical NSCs show bi-directional effects, including increased NSC self-renewal and neurogenesis, suppression of NSC differentiation, and stimulation of blood-brain barrier formation (Shen et al., 2004; Weidenfeller et al., 2007). Also, endothelial cells seeded onto embryonic NSC-derived neurospheres (NS) in 3-dimensional cultures attach and migrate into the NS, further supporting interactions between neural and vascular elements (Milner, 2007).

Less is known about how brain injury influences the SVZ niche. Stroke in neonatal and adult rodents increases striatal SVZ neurogenesis and stimulates neuroblast migration to injury, resulting in low-level cell replacement (Arvidsson et al., 2002; Parent et al., 2002; Plane et al., 2004). Stroke-induced neurogenesis may arise from changes in the SVZ niche that promote proliferation, along with injury cues that attract migrating neuroblasts and stimulate their differentiation. Supporting this idea are findings that SVZ angiogenesis and neurogenesis increase after cortical thermocoagulation lesion (Gotts and Chesselet, 2005). Stroke also stimulates neuroblast migration to peri-infarct cortex alongside blood vessels (Ohab et al., 2006). Moreover, administration of the angiogenesis inhibitor endostatin after experimental stroke decreases both angiogenesis and neurogenesis, suggesting that angiogenesis is necessary for stroke-induced neurogenesis (Ohab et al., 2006). Consistent with these findings, co-cultures of SVZ NSCs with intact or ischemia-altered endothelial cells reveal that intact endothelial cells increase NSC proliferation, while those from the ischemic border stimulate neuronal differentiation (Teng et al., 2008).

To further investigate how normal or injured endothelial cell secreted-factors regulate postnatal SVZ NSCs, we co-cultured SVZ-derived NS with endothelial cells in a non-contact system or in endothelial cell-conditioned media (ECCM) using intact or oxygen-glucose deprived (OGD, an *in vitro* stroke model) endothelial cells. We also expanded NS in conditioned media collected from intact or OGD-treated endothelial cells. We found that expansion in intact ECCM increased NS production and cell proliferation. NS exposed to intact endothelial cells or ECCM generated more immature-appearing neurons, whereas OGD-treated endothelial cells stimulated neuroblast chain migration and neuronal differentiation. Intact endothelial cell co-culture or ECCM, as well as OGD-treated ECCM, also influenced glial morphology and numbers. These results suggest that intact endothelial cell-secreted factors maintain SVZ NSCs in an immature state, and that injury stimulates endothelial cells to support neuronal migration and differentiation.

MATERIALS AND METHODS

Primary Neurosphere (NS) Culture

Animal protocols were approved and procedures performed in accordance with University of Michigan Committee on Use and Care of Animals policies. NS cultures were prepared as described, (Wang et al., 2005) with slight modifications. Postnatal day 15 (P15) CD-1 mice (Charles River) were anesthetized with CO₂, decapitated, brains removed, and placed into ice cold Opti-mem. Forebrain containing the striatal SVZ was cut into two coronal slices and the SVZ dissected out, minced and dissociated with trypsin. SVZ cells (6×10^3 – 6×10^4 /well) were cultured in serum-free media (SFM) containing growth factors [Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, Invitrogen, Carlsbad, CA), 20 ng/ml epidermal growth factor

(EGF), 10 ng/ml basic fibroblast growth factor (bFGF) and 2 µg/ml heparin (all from Sigma-Aldrich, St. Louis, MO), and a defined hormone and salt mixture (100 µg/ml transferrin, 25 µg/ml insulin, 60 µM putrescine, 30 nM sodium selenite and 20 nM progesterone). Primary NS were cultured for 6 d, then picked and re-plated for differentiation in polyornithine-coated 24-well plates or mechanically dissociated and passaged to form secondary NS. Unless otherwise described, primary NS were differentiated for 7 d in DMEM/F12 plus hormone mixture and 1% fetal bovine serum (FBS). Half of the medium was replaced every 3 d.

Endothelial Culture

Primary mouse brain endothelial cells or the brain endothelial cell line (bEnd.3) were cultured as previously described (Andjelkovic et al., 2003). Primary endothelial cells were cultured from 4- to 6-week-old CD-1 mouse brain microvessels in DMEM plus 10% inactivated fetal calf serum (Invitrogen), 2.5 g/mL heparin, 20 mM HEPES, 2-mM glutamine, antibiotic/antimycotic (Invitrogen), and endothelial cell growth supplement (BD Bioscience, San Jose, CA) using collagen IV-coated six-well plates. The bEnd.3 line was purchased from ATCC (American Type Culture Collection, Manassas VA). Cells were plated and grown in the recommended media (DMEM, 10% FBS, $1 \times$ AA, 2 mM glutamine) in 10% CO₂.

Co-culture Experiments

In experiment one, NS were cultured with primary mouse brain endothelial cells in a non-contact manner, similar to that described by Shen et al (Shen et al., 2004). Endothelial cells were weaned from 10% serum to serum-free media (SFM) over 3 d for co-culture with NS. SVZ cells plated on transwell inserts (40 µm pore size, Corning, Lowell, MA) at 5×10^4 cells/insert were expanded alone for 3 d (Figure 1). NS-containing inserts were then placed into wells containing endothelial cells in SFM. Controls included NS grown in SFM on membranous inserts with NIH3T3 cells or no cells plated below the insert. NS expansion continued for another 3 days. At 6 d, similarly sized NS were picked and re-plated for differentiation.

Conditioned Media Differentiation Experiments

Conditioned media (CM) was collected from primary mouse brain endothelial or NIH3T3 cells grown in DMEM with 10% normal calf serum (NCS). CM was collected approximately 24 h after application to cells, filtered, and stored at -20°C . For experiment two (Figure 1), NS were plated at 6×10^4 cells/60mm dish and expanded in SFM for 6 d, then were picked and re-plated on polyornithine-coated 24-well plates for differentiation in 100% or 50% (diluted 1:1 with DMEM) CM from endothelial or 3T3 cells, or in unconditioned (control) media containing matching 10% or 5% NCS. NS were differentiated in CM for 7 d, with half replaced every 3 d.

Oxygen-Glucose Deprivation (OGD)

All OGD experiments were performed using the bEnd.3 line as previously described (Andjelkovic et al., 2003). Confluent bEnd.3 cells were transferred into a temperature-controlled ($37^{\circ} \pm 1^{\circ}\text{C}$) anaerobic chamber (Coy Laboratory, Grass Lake, MI) containing 5% CO₂, 10% H₂ and 85% N₂. The medium was replaced with deoxygenated glucose-free, serum-free DMEM and cells maintained in anaerobic conditions for 5 h. OGD-exposed cells were removed from the chamber, the medium replaced with fresh serum-free DMEM and returned to normoxic conditions (5% CO₂/95% air) for up to 24 h (re-oxygenation). Control cultures were not exposed to OGD. Serum-free CM was collected from injured endothelial cells at 6, 12 or 24 h post-OGD (OGD-ECCM), or from intact endothelial (ECCM) or NIH3T3 cells (3T3CCM). Unconditioned media served as an additional control. Media were filtered and stored at -20°C .

For experiment three (Figure 1), SVZ cells were plated at 1.0×10^4 cells/well in 12-well plates and expanded alone in SFM containing doubly concentrated growth factors for 24 h. An equal volume of conditioned (or unconditioned) media was then added to each well and NS expansion continued for 6 d total. Primary NS were picked and re-plated for 4 or 7 d differentiation in 1% FBS, or were passaged and re-plated in SFM (unconditioned) to form secondary NS.

To assess migration, primary NS were grown alone for 5 d, then picked and re-plated in Matrigel (Figure 1, Experiment 4) following published methods (Katakowski et al., 2005). NS were incubated at 37°C for 30 min to allow the Matrigel to solidify. CM or control media was added and NS placed in a 37°C/5% CO₂ incubator. Phase-contrast images were captured at 24 h and 3 d post-plating, and then NS were fixed and immunostained.

Immunofluorescence Histochemistry

Cultures were fixed with 4% paraformaldehyde and immunostained using antibodies to neurons (1:400 anti-mouse Map2abc, Sigma-Aldrich; 1:1000 anti-rabbit β -III-tubulin, Covance, Princeton, NJ; 1:800 anti-rabbit doublecortin [DCx], (Parent et al., 2002)), astrocytes (1:500 anti-mouse glial-fibrillary associated protein [GFAP], Sigma-Aldrich), radial glia (1:100 anti-mouse ZRF-1, Zebrafish Information Network [ZFIN], Eugene, OR), oligodendrocytes (1:600 anti-rat myelin basic protein [MBP], Millipore [Chemicon], Billerica, MA), neural progenitors (1:100 anti-mouse nestin; 1:500 anti-rabbit NG2, both from Millipore), proliferating cells (1:1000 anti-rabbit Ki67, Vector Labs, Burlingame, CA), dying cells (1:1000 anti-rabbit activated caspase-3, BD Biosciences), or endothelial cells (1:400 anti-rabbit Glucose transporter-1 [Glut-1], Millipore). Cells were washed with PBS, blocked, and incubated in primary antibody overnight at 4°C. After washes, cells were incubated in secondary antibody (1.5 h, room temperature). Secondary antibodies were goat-anti-rabbit Alexa 594, goat-anti-mouse Alexa 488, and goat-anti-rat Alexa 488 (Invitrogen). Cells were washed and nuclei counterstained with bisbenzamide.

Microscopy, Image Analysis and Statistics

Cultures were analyzed using a Leica DMIRB inverted microscope (Wetzlar, Germany) and SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI). NS numbers were counted and diameters measured in 2 wells/condition/experiment (≥ 4 separate experiments) under phase microscopy; differentiated cultures were imaged under epifluorescence. NS of similar size and density were selected based on bisbenzamide nuclear labeling and a minimum of 3 NS/condition were photographed for quantification using a 20X objective. Images were imported into Adobe Photoshop v. 7.0 (Adobe Systems, San Jose, CA) or NIH ImageJ for analysis. Images of β -III-tubulin immunoreactivity with the examiner blinded to condition were used for both process number and length measurements, and branching analyses. The lengths of β -III-tubulin+ neuronal processes were measured using ≥ 3 NS/condition for at least 3 independent experiments. For branch analyses, the examiner first counted the total number of branch points for each cell, and then the numbers of primary, secondary and tertiary branches extending from the cell body were quantified for each β -III-tubulin-positive cell. The main process extending from the cell body was considered the primary branch, while processes splitting from the main branch were counted as secondary branches. Processes splitting from the secondary branches were counted as tertiary branches. Cells with only one process extending from the cell body were given a zero for the number of branch points, secondary branches and tertiary branches. Oligodendrocytes were quantified in ≥ 3 separate experiments by counting MBP+ cells (≥ 3 fields/condition under a 20X objective). Proliferating (Ki67+) or dying (activated caspase-3+) cells were quantified similarly. Migration in Matrigel cultures was analyzed by measuring the distance from the NS center to the ends of the 3 longest chains/NS for 3 or more NS/condition/experiment using ImageJ. Analysis of variance (ANOVA) with Fisher's Protected Least Squares Differences post-hoc test was used to compare group

differences with Statview software (Adept Scientific, Hertz, UK). Results are presented as mean $\pm$ standard error of the mean (SEM) and considered significant when $p \leq 0.05$.

RESULTS

Endothelial cell-derived factors influence NS-derived neurons and glia

Interactions between components of the NSC microenvironment regulate persistent forebrain neurogenesis (Ninkovic and Gotz, 2007). Evidence suggests that endothelial cells are key elements in the SVZ neurogenic niche (Gotts and Chesselet, 2005; Thored et al., 2007), but the potentially broad range of endothelial influences on postnatal SVZ NSCs are poorly understood. To directly examine these influences, we first expanded SVZ-derived NS with endothelial cells in non-contact (transwell) co-cultures (Figure 1, Experiment 1). Control NS were expanded alone or co-cultured with NIH3T3 cells. Co-culture of primary NS with endothelial cells for up to 3 days did not increase NS size or numbers after expansion for 6 days total (data not shown). To examine whether endothelial cell co-culture modulates neurogenesis, we differentiated co-cultured NS for 7 days in untreated medium and identified neurons by immunostaining for β -III-tubulin or DCx. Differentiation in normal media after co-culture did not increase neuron production; however, the morphology of β -III-tubulin-expressing neurons was altered as those derived from endothelial co-cultured NS were more clustered and displayed significantly shorter processes, averaging less than half the length of neuronal processes in control cultures (Figure 2A–C; $p < 0.01$; F, 16.93, DF, 2, 38). Similar results were seen for DCx-labeled immature neurons, with those exposed to endothelial cells or ECCM showing substantially more clustering and less migratory or complex morphology (Supplemental Figure 1A–D).

Next we sought to determine if endothelial cell co-culture influences NSCs or glia in SVZ-derived NS. We first examined nestin immunoreactivity and found that NS cultured alone and differentiated in 1% FBS without mitogens contained few nestin-positive cells after 7 days (Figure 3C). NS co-cultured with endothelial cells and then differentiated alone in the same media as controls, in contrast, showed many nestin-immunoreactive cells with long radial processes and stronger nestin expression than in either of the control conditions (Figure 3A–C). Similar results were found using the ZRF-1 antibody that labels radial glia in zebrafish (Trevarrow et al., 1990), in that immunoreactivity was not present in control cultures but was present in long, radial processes in endothelial co-cultured NS (Supplemental Figure 1E–H). We next immunostained for GFAP to identify astrocytes. GFAP-positive cells showed altered morphology in NS co-cultured with endothelial cells, compared to co-culture with 3T3 cells or NS cultured alone, prior to differentiation (Figure 3D–F). GFAP-positive cells derived from endothelial co-cultured NS contained numerous processes extending off of each cell, resembling reactive astrocytes (Figure 3D) while those from controls typically showed a larger, flatter morphology (Figure 3E–F). Taken together, these findings suggest that a transient exposure of NS to endothelial cells during expansion alters NSCs, glia and neuroblasts in the cultures, as all progeny appear more immature after subsequent differentiation.

SVZ NSCs cultured as NS generate neurons, astrocytes and oligodendrocytes. Postnatal SVZ progenitors also give rise to oligodendrocytes *in vivo* (Levison and Goldman, 1993). We therefore examined the influence of endothelial cell-secreted factors on oligodendrocytes generated by SVZ-derived NS. We found that MBP-positive progeny of NS exposed to endothelial cell co-culture during expansion appeared smaller in size and displayed less branching than those from control cultures (Supplemental Figure 2). Quantification of MBP-positive cells showed no difference in the number of oligodendrocytes produced from endothelial co-cultured NS compared with NS expanded alone, although 3T3 cell co-cultured NS gave rise to significantly fewer oligodendrocytes ($p < 0.05$; F, 3.66, DF, 2, 60; Supplemental Figure 2).

A recent study suggested that NSCs could transdifferentiate into endothelial cells after exposure to endothelial cells in co-culture (Wurmser et al., 2004). We performed immunostaining to identify endothelial cells using anti-Glut-1 antibody and found no immunoreactivity in NS that were expanded with primary endothelial cells (data not shown). Endothelial cells from co-culture were included as a control and all of these cells expressed Glut-1 (data not shown). Also, no cells in NS cultures showed an endothelial cell-like morphology. We therefore found no evidence using primary endothelial cell/NS transwell co-cultures that NS-derived cells become endothelial-like cells under these conditions.

We next examined whether ECCM modulates SVZ-derived NS cultures. Because ECCM contains serum, we could not add it to expanding NS without inducing their differentiation. Instead, we differentiated untreated NS in ECCM, or in 3T3CCM or unconditioned medium as controls (Figure 1, Experiment 2). NS differentiated in ECCM gave rise to clusters of β -III-tubulin-immunoreactive neurons with much shorter processes than controls (Figure 2D, E). Measurements revealed that NS differentiated in ECCM (50 or 100%) generated neurons with processes $\sim 1/3$ – $1/2$ the length of controls ($p < 0.01$; F, 4.66, DF, 5, 36; Figure 2F).

We also performed nestin and GFAP immunostaining on NS after differentiation in ECCM. Nestin-immunoreactivity appeared strong in radial processes of 100% ECCM-differentiated NS (Figure 3G). Smaller numbers of cells from 3T3CCM-differentiated NS also strongly expressed nestin, but these cells showed a flat, non-radial morphology (Figure 3H). Very little nestin expression was detected in serum controls and rare labeled cells, when present, resembled normal astrocytes (Figure 3I). GFAP-positive cells from ECCM-differentiated NS resembled the nestin-expressing cells, with strong staining of long processes (Figure 3J). GFAP-positive cells from both 3T3CCM and serum controls displayed more typical astrocyte morphology (Figure 3K–L). Experiments performed with 50%-diluted ECCM (and 50% 3T3CCM or 5% serum control) showed similar results (data not shown). The morphology of oligodendrocytes was also examined after NS differentiation in ECCM. NS differentiated in 50 or 100% ECCM produced smaller MBP-positive oligodendrocytes with less branching than in control cultures (Figure 3M–P). The number of MBP-positive cells was significantly increased in 50% ECCM-differentiated NS compared with all other conditions, but the total numbers of oligodendrocytes per NS was very low in all conditions (Figure 3Q, $p \leq 0.05$; F, 2.48, DF, 5, 36).

Previous studies examining the influence of endothelial cells on embryonic NSCs found increased self-renewal after co-culture of these two cell types (Shen et al., 2004). The effects of ECCM on NS expansion or self-renewal could not be tested in the current experiments, however, because the endothelial cells were cultured in serum. Also, endothelial cells tolerated co-culture with NS in SFM only up to three days. We describe additional experiments below evaluating effects of endothelial cell-secreted factors on NSC self-renewal using serum-free ECCM.

Endothelial-derived factors increase NS expansion

Experimental stroke increases neurogenesis, expands the SVZ and induces angiogenesis in peri-infarct regions (Arvidsson et al., 2002; Chen et al., 2004; Ohab et al., 2006; Parent et al., 2002; Wang et al., 2008; Wang et al., 2004; Zhang et al., 2001). The correlation between stroke-induced angiogenesis and neurogenesis led us to question whether stroke-injured endothelial cells regulate SVZ NSCs. We therefore used OGD, an established *in vitro* stroke model, to induce ischemia-like injury in endothelial cells (Andjelkovic et al., 2003; Hu et al., 2006; Kapinya et al., 2002; Keep et al., 2005; Perez-Pinzon et al., 1995). SFM conditioned for 6 or 12 h by intact NIH3T3 or endothelial cells, or OGD-exposed endothelial cells (labeled 6 h ECCM, 12 h ECCM, etc.) was tested for its effects on expanding NS cultures (Figure 1, Experiment 3). Primary NS were expanded for 24 hours in SFM and then for 5 days in intact

ECCM, 3T3CCM (or unconditioned media as an additional control) or OGD-ECCM. We found a significant increase in the number of NS produced after expansion in intact 12 h ECCM compared with control conditions (Figure 4A, $p \leq 0.01$; F, 2.29, DF, 6, 28). OGD-ECCM did not affect NS expansion.

Some NS were then differentiated while others were passaged to examine self-renewal. No significant difference was found in the number of secondary NS produced between conditions, but a trend ($p=0.08$) toward increased secondary NS production was seen for primary NS expanded in 12 h ECCM (Figure 4B). No significant difference from controls emerged in the primary or secondary NS size after expansion in intact ECCM or OGD-ECCM (data not shown). Next we evaluated the effects of CM on cell proliferation. After 4-day differentiation, we found significantly more Ki67-positive cells in ECCM-treated NS compared with controls or OGD-ECCM (Figure 4C–I). Cell death assayed by activated caspase-3 immunostaining showed no difference between ECCM, OGD-ECCM or control conditions ($p=0.71$; F, 0.65, DF, 7, 89; Supplemental Figure 3G–M). Together, these data indicate that factors secreted from intact endothelial cells increase SVZ NSC expansion, proliferation and perhaps self-renewal without altering cell death.

Endothelial-secreted factors alter neuronal maturation and glial morphology

In our initial experiments, NS expansion with primary endothelial cells before differentiation or differentiation of NS in ECCM altered the morphology of neuronal and glial progeny. To determine whether exposure of NS to serum-free ECCM or OGD-ECCM before differentiation would exert similar effects, we expanded NS for 5 days in serum-free conditioned media from intact or OGD-treated endothelial cells, or control 3T3 cells, and then differentiated in normal medium for 4 or 7 days (Figure 1, Experiment 3). After 4 day differentiation, NS expanded in ECCM contained MAP2abc-positive cells with short or no processes while labeled cells in controls had a mix of short and medium processes (Supplemental Figure 4E–H). NS expanded in OGD-ECCM gave rise to MAP2abc-positive neurons with much longer processes than controls (Supplemental Figure 4E–H). After 7 day differentiation, β -III-tubulin immunostaining revealed that neurons from intact ECCM-expanded NS continued to exhibit shorter processes, while processes of neurons from OGD-ECCM-expanded NS remained longer than controls (Figure 5A–D).

The number of processes/cell and length of each process was measured in similar density fields. Quantification of process length (Figure 5E) confirmed that neurons from intact 6-hour ECCM contained significantly shorter processes than controls ($p=0.03$ vs. UNCOND; F, 11.76, DF, 2, 36), as did 12-hour ECCM ($p<0.05$ vs. all controls; F, 9.09, DF, 3, 44). The analysis also found that processes from 6- and 12-hour OGD-ECCM were significantly longer than controls [$p \leq 0.01$ vs. all controls; F, 13.95 (6 hour), 13.06 (12 hour), DF, 2, 32 (for 6 & 12 hour)]. Branching analyses on experiments using conditioned media collected for 6 or 12 hours (Table 1) revealed significant differences in numbers of branch points (6 hour: $p<0.01$; F, 4.63; DF, 3, 36; 12 hour: $p<0.0005$; F, 7.69; DF, 3, 38), secondary branches (6 hour: $p<0.005$; F, 5.84; DF, 3, 36; 12 hour: $p<0.01$; F, 4.44; DF, 3, 38) and tertiary branches (6 hour: $p<0.05$; F, 3.02; DF, 3, 36; 12 hour: $p=0.001$; F, 6.49; DF, 3, 38). There was more branching (in each of the indices) in the 12 hour OGD-ECCM group compared to the unconditioned, 12 hour ECCM and 12 hour 3T3CCM groups. The 6 hour OGD-ECCM group also had more branching in some of the indices compared to the 6 hour ECCM and 3T3CCM groups, although the magnitude of this effect was less than the 12 hour OGD-ECCM group. We observed no difference between conditions in numbers of processes per cell (data not shown) or the numbers of neurons/field (Table 1).

Due to the changes in progenitors and glia seen in our initial experiments with NS/endothelial cell co-culture or NS differentiation in serum-containing ECCM, we examined whether similar

changes occurred after NS expansion in serum-free intact- or OGD-ECCM. SVZ-derived NS were expanded in serum-free CM, differentiated for 4 days and immunostained for nestin. Similar to the earlier experiments, we found that nestin-positive cells from ECCM-expanded NS displayed long radial processes (Figure 5H, arrowheads), whereas less nestin was expressed in unconditioned control cultures and nestin-immunoreactive cells in 3T3CCM controls did not show this morphology (Figure 5F–H). NS expanded in OGD-ECCM yielded a mixed population of nestin-positive cells, some with numerous short processes (Figure 5I, arrows) and others with lighter expression and long processes (Figure 5I, arrowheads). After 7 days, striking differences in morphology persisted in GFAP-immunoreactive cells (Figure 5J–M). NS differentiated in ECCM, and to a lesser extent OGD-ECCM, also expressed the radial glia cell marker RC2, which was absent in control cultures (Supplemental Figure 4A–D).

We next evaluated the influence of intact- or OGD-ECCM on SVZ NS-derived oligodendrocytes. Few oligodendrocytes were produced with no clear difference in the number of MBP-positive cells generated from intact- or OGD-ECCM expanded NS or in their morphology (Supplemental Figure 3A–F). NS in some experiments were also stained with antibodies to identify endothelial cells (Glut-1). We found no evidence of transformation of SVZ-derived NSCs into endothelial cells (data not shown).

Factors from OGD-ECCM stimulate chain migration

We next examined whether intact or OGD-treated ECCM influence neuronal migration from SVZ-derived NS. NS were expanded for 5 days and plated in Matrigel to promote chain migration. Conditioned medium collected after 6 or 12 hours was added to the Matrigel cultures, and NS were cultured for three more days (Figure 1, Experiment 4). NS incubated with intact ECCM showed some chain migration at 24 hours that appeared similar to controls (Figure 6A, B, D, E). NS exposed to OGD-ECCM, in contrast, exhibited much more migration (Figure 6C, F). After 3 days in Matrigel, NS in OGD-ECCM contained significantly longer chains than controls (Figure 6H, $p \leq 0.05$ for 6-hour OGD-ECCM, $p \leq 0.01$ for 12-hour OGD-ECCM), while NS in intact ECCM remained similar to controls or trended toward less migration (Figure 6H). Quantification of chain length at 3 days probably underestimated the migratory effect, as differences were even more apparent at 24 hours (Figure 6A–F). Immunofluorescence double-labeling showed that the long chains extending from OGD-ECCM-treated NS were MAP2abc-positive neuroblasts (green), with few GFAP-positive astrocytes present (Figure 6G). Together these findings indicate that OGD-ECCM stimulates neuroblast migration.

DISCUSSION

These experiments suggest that factors secreted by intact and OGD-treated endothelial cells influence the proliferation, migration, and differentiation of postnatal forebrain SVZ-derived NSCs *in vitro*. OGD treatment of endothelial cells in some instances produced CM with very different effects than that from intact ECCM (Table 2). We found that intact ECCM promotes NS production and cell proliferation, and endothelial cell co-culture or ECCM yielded neurons that appeared more immature than controls. Progenitor or glial cells derived from these NS also displayed altered morphology, suggestive of radial glia or reactive astrocytes. Furthermore, we found increased oligodendrocyte production during exposure to ECCM under certain conditions, and more immature-appearing oligodendrocytes were generated after NS co-culture with endothelial cells or differentiation in ECCM. Finally, NS expanded in OGD-ECCM displayed faster chain migration and neuron process outgrowth, effects opposite to those of intact endothelial cell co-culture or ECCM.

Our data support the idea that intact endothelial cells secrete factors that maintain NSCs in a stem cell-like state or slow the differentiation of their progeny. We observed a significant

increase in NS production after expansion in ECCM. In addition, expression of the NSC marker nestin and the radial glial markers ZRF1 and RC2 increased after NS co-culture with endothelial cells, expansion in ECCM or differentiation in ECCM. Endothelial cells also altered the morphology of nestin- and GFAP-expressing cells to a more radial glia-like state, consistent with a previous report (Weidenfeller et al., 2007), and suggestive of a more undifferentiated or progenitor-like condition. We also found increased cell proliferation in differentiating NS that had been expanded in ECCM, similar to studies involving co-culture of embryonic or adult NSCs with endothelial cells (Shen et al., 2004; Teng et al., 2008). In terms of neuronal progeny, differentiating neurons appeared more immature than controls regardless of whether we co-cultured NSCs and endothelial cells during NS expansion, expanded NS in ECCM, or differentiated them in ECCM. The first two conditions suggest a priming effect because exposing NS solely prior to differentiation still influenced their subsequent development. Weidenfeller and colleagues also found neurons with reduced process number and length from NS differentiated after being expanded in co-culture with endothelial cells (Weidenfeller et al., 2007). Lastly, we noted that MBP-immunoreactive oligodendrocytes from endothelial cell co-culture or ECCM differentiation were smaller and displayed less branching, suggesting that they were more immature than those from control cultures. Taken together, these data suggest a model in which endothelial cells in the NSC niche regulate stem cell behavior to maintain the NSC population and slow differentiation of their progeny that remain within the niche.

Several recent studies have examined the interaction of intact endothelial cells and NSCs using NS cultures. Two focused on embryonic NSCs isolated from the developing cortex and another used postnatal rat hippocampal NSCs, whereas we examined the effects of endothelial cells on postnatal mouse NSCs isolated from the forebrain SVZ (Guo et al., 2008; Shen et al., 2004; Weidenfeller et al., 2007). Despite the differences in age and location from which the NSCs were derived, some of our results were similar. In addition to radial glia-like progenitor morphology and more immature-appearing neurons (Weidenfeller et al., 2007), several groups found increased nestin expression immediately after co-culture of NSCs and endothelial cells, and one commented on increased clone size (Guo et al., 2008; Weidenfeller et al., 2007).

Some of our data supporting endothelial cell influences on NSCs differ from those described previously. The initial work exploring endothelial cell effects on SVZ NSCs involved SVZ explants cultured with endothelial cells via direct contact or separated by an insert (Leventhal et al., 1999). The authors found that endothelial cell-secreted factors increased neuronal migration out of explants, and provided evidence that brain-derived neurotrophic factor (BDNF) produced by endothelial cells was the critical factor mediating the effects. The explant preparation is more heterogeneous than NS cultures, however, and the investigation was limited to effects on neuroblasts migrating out of the explants without exploring the stem or progenitor cell populations. Other reports also described increased neurogenesis after co-culturing embryonic forebrain or adult hippocampal NSCs with endothelial cells (Guo et al., 2008; Shen et al., 2004). We did not find increased neurogenesis in any of our culture conditions, perhaps because of differences in the age or location from which our NSCs were derived compared to the previous studies. Our findings of altered morphology and increased production of oligodendrocytes after differentiation in diluted ECCM are novel. The specific conditions required for the latter effect in our experiments, 50% diluted ECCM, may relate in part to the amount of serum (5%) in the media.

A number of studies have identified correlations between stroke-induced angiogenesis and neurogenesis (Chen et al., 2004; Gotts and Chesselet, 2005; Ohab et al., 2006; Sun et al., 2003; Taguchi et al., 2004; Thored et al., 2007; Wang et al., 2004), but the direct effects of injured endothelial cells on NSCs are relatively unexplored. We therefore examined the influence of stroke-injured endothelial cells, using OGD as an *in vitro* stroke model, on SVZ NSC expansion, migration, and differentiation. OGD-ECCM, unlike intact ECCM, did not

stimulate NS expansion. Instead, OGD-ECCM significantly promoted chain migration and increased neuronal process outgrowth compared with intact ECCM or controls. Using co-culture of normal adult SVZ stem cells with endothelial cells from the ischemic border, a very recent study found that injured endothelial cells increased neuron production from the cultures while intact endothelial cells promoted cell proliferation (Teng et al., 2008). In contrast, we did not find increased neurogenesis but instead observed more rapid migration and differentiation of neuroblasts exposed to OGD-ECCM. Nonetheless, it is clear from these *in vitro* studies that intact and injured endothelial cells differentially influence SVZ-derived NSCs.

Interestingly, a recent *in vivo* study using a cortical stroke model reported evidence of neuroblast clusters adjacent to newly formed endothelial cells in the peri-infarct region, as well as neuroblasts migrating alongside blood vessels into peri-infarct cortex (Ohab et al., 2006). Angiogenesis inhibitor treatment after stroke decreased the number of new endothelial cells and neuroblasts in the penumbra. Treatment with a pro-angiogenic factor, angiopoietin-1, increased neuroblast numbers near the infarct, again in close proximity to endothelial cells (Ohab et al., 2006). These data suggest that peri-infarct endothelial cells may provide cues that attract neuroblasts to injured regions. This idea fits well with our results, in that OGD-injured ECCM promoted neuroblast chain migration and stimulated neuronal process outgrowth. These findings provide interesting insight into the function of intact and injured endothelial cells in the neurogenic niche. Under normal circumstances, endothelial cells appear to provide factors that maintain the niche in a more proliferative and undifferentiated state. After injury, however, these factors may be down-regulated and other factors secreted by injured endothelial cells induce NSCs to migrate out of the SVZ and differentiate more rapidly. Our hypothesis is that endothelial cells in the SVZ, which is not damaged in the aforementioned stroke models, are activated to increase proliferation and self-renewal of the NSCs, while endothelial cells in the infarct and peri-infarct regions down-regulate these factors and instead provide cues to attract NSCs to migrate to the injury and differentiate rapidly to potentially replace dying cells.

The specific endothelial cell-secreted factors that influence neurogenesis are largely unknown. As mentioned earlier, endothelial-derived BDNF likely promotes neurogenesis and neuroblast migration (Leventhal et al., 1999). Additional agents have been examined, including retinoic acid, forskolin, fetal bovine serum, or leukemia inhibitory factor plus vascular endothelial growth factor (VEGF), without measurable effects on *in vitro* neurogenesis from embryonic NSCs (Shen et al., 2004). VEGF, a pro-angiogenic factor expressed by endothelial cells, remains a prime candidate as it promotes normal and stroke-induced neurogenesis (Meng et al., 2006; Sun et al., 2003), mediates adult NSC proliferation and neuronal differentiation *in vitro* via up-regulation in endothelial cells isolated from the ischemic border (Teng et al., 2008), and enhances NSC survival (Wada et al., 2006). Additional pro-angiogenic factors such as stem cell factor (Jin et al., 2002), matrix metalloproteinase-2 and 9 (Wang et al., 2006), and pigment-epithelial-derived factor (Ramirez-Castillejo et al., 2006) also influence NSCs in a variety of ways and are potential candidates. In terms of OGD-exposed endothelial cell stimulation of neuroblast migration in particular, similar results appear when CM from hypoxia-exposed astrocytes is applied to NSCs (Xu et al., 2007). Enhanced migration in this setting is associated with upregulation of VEGF and other chemokines (stem cell factor, stromal-derived factor-1 and monocyte chemoattractant protein-1), and the migration is partially suppressed by inhibiting each of these chemokines in hypoxia-exposed astrocyte CM (Xu et al., 2007). Taken together, this work suggests that multiple molecular pathways are involved in the pleiotrophic effects of intact or injured endothelial cells on SVZ NSCs.

Our findings suggest that endothelial cells are an important component of the neurogenic niche. They appear to maintain postnatal SVZ NSCs in their stem cell-like state under normal conditions, whereas injured endothelial cells prompt NSC-derived neuroblasts to migrate and

differentiate, potentially serving as an endogenous repair mechanism for neuronal replacement after injury. Identifying the key mediators involved in these endothelial influences therefore may offer novel targets for brain restorative therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic showing neurosphere (NS) culture experiments

For all experiments, the subventricular zone (SVZ) was dissected from P15 mice, trypsinized, and plated as single cells in serum-free media containing EGF and bFGF. For experiment one, SVZ-derived NS were expanded alone for 3 d in transwell inserts, and inserts were transferred to wells containing primary endothelial cells in the same serum-free media with mitogens. NS were co-cultured (co-cx) with endothelial cells for 3 d, and then differentiated alone for 7 d in media containing 1% serum. In experiment two, NS were expanded alone for 6 d and then were differentiated in conditioned media (CM) containing 5 or 10% serum for 7 d. Experiment three consisted of NS expanded in normal medium for 24 h, and then in serum-free CM from normal or oxygen-glucose deprived (OGD) endothelial cells for 5 d. Primary NS were analyzed and then differentiated for 4 or 7 d. For experiment four, NS were expanded alone for 5 d and then plated in 3-dimensional matrigel culture with serum-free conditioned media for 3 d to assess chain migration.

Figure 2. Endothelial cell-secreted factors maintain neurons in an immature state

A–C: SVZ-derived NS were expanded in non-contact co-culture with primary endothelial cells, NIH3T3 cells or alone, and then were differentiated alone for 7 d. β -III-tubulin+ neurons from NS expanded with endothelial cells subsequently displayed short processes and cells tended to cluster after differentiation (A), whereas those expanded with 3T3 cells or alone extended longer processes that contacted other neurons (B). Quantification of neuron process length revealed that neurons derived from NS co-cultured with endothelial cells had significantly shorter processes (C, $*p < 0.01$ versus the control groups). D–F: NS differentiated in (10% serum-containing) ECCM from primary mouse endothelial cell cultures showed neurons that were more clustered and had shorter processes than controls (D, E). Measurement of process length revealed that β -III-tubulin+ neurons differentiated in 100% or 1:1 diluted (50%) ECCM averaged about one-third the length of controls (F; $*p < 0.01$ versus both control groups). Scale bar: 50 μ m.

Figure 3. Endothelial cell-secreted factors alter glial morphology

A–F: SVZ-derived NS expanded with primary endothelial cells and then differentiated for 7 d showed increased nestin expression in cells with long processes (A) compared to controls (B, C). GFAP immunostaining under the same conditions revealed cells with a more reactive astrocyte-like morphology (D) compared to the more typical flat GFAP+ astrocytes from control co-cultures (E, F). G–L: After differentiation in ECCM, nestin is expressed in cells with long processes (G) whereas its expression level is low and present in cells of typical astrocyte morphology in 3T3CCM-differentiated NS (H) or minimal in unconditioned (UNCOND) controls (I). GFAP+ cells from ECCM-differentiated NS (J) extend more numerous and longer processes than controls (K, L) and resemble those that express nestin

(G). M–Q: NS were differentiated in undiluted (100%; M) or half-diluted (50%; O) ECCM or similarly diluted 3T3 cell CM (N, P), and oligodendrocytes identified by MBP immunostaining. ECCM-differentiated NS contained mostly small MBP+ oligodendrocytes with less branching (M, O) than controls (N, P), and appear similar to those seen in NS from co-culture with endothelial cells (not shown). NS differentiated in 50% ECCM produced more MBP+ oligodendrocytes than all of the other conditions (O, Q, * $p < 0.01$ vs. all other conditions). Scale bars: 100 μm in A (for A–C; M–P), 50 μm in D (for D–L).

Figure 4. Endothelial cell-secreted factors increase neurosphere production and SVZ neural stem cell proliferation

A–B: SVZ-derived NS were expanded in CM from intact (ECCM) or OGD-exposed endothelial cells (OGD-ECCM) or control media (3T3CCM or unconditioned). The number and size of NS was obtained after 6 d of expansion and data graphed as percent of unconditioned control (CM/unconditioned $\times$ 100%). Expansion in intact 12 h ECCM led to significantly more primary NS produced than in other conditions (A; $*p \leq 0.01$ 12 h ECCM vs. 12 h 3T3CCM, 6 h 3T3CCM and unconditioned). After NS expansion in CM, cells were dissociated, re-plated in SFM and then expanded to form secondary NS. Secondary NS number was increased, though not significantly in 12 h ECCM compared with controls (B; $p = 0.08$ for 12 h ECCM vs. 12 h 3T3CCM). C–I: Primary NS were expanded in CM for 6 d, differentiated for 4 d, and then immunostained for Ki67. NS expanded in 3T3CCM (C, F) or OGD-ECCM (E, H) contained very few Ki67+ cells. NS expanded in intact ECCM contained significantly more Ki67+ cells (D, G) compared to all other conditions. Quantification of similar density NS revealed that those from intact ECCM contained ~3 times as many Ki67+ cells as those from OGD-ECCM, and >1.5-fold more than controls (I, $*p < 0.01$ for 6 h ECCM vs. unconditioned; 12 h ECCM vs 12 h 3T3CCM, 12 h OGD-ECCM or unconditioned). 6 h OGD-ECCM group trended toward decreased cell proliferation compared to controls ($p = 0.08$ for 6 h OGD-ECCM vs 6 h 3T3CCM). Scale bar: 100 μ m.

Figure 5. Endothelial cell-secreted factors alter neuron and glia maturation

A–E: After a 7 day differentiation, NS expanded in intact ECCM contained β -III-tubulin-immunoreactive neurons with very short or absent processes (arrows, C) compared to controls (A–B). Neurons derived from NS expanded in OGD-ECCM displayed much longer processes than in the three other groups, characteristic of more mature neurons (D, arrowheads).

Quantification of process length (graphed as a percent of unconditioned media control: CM/unconditioned $\times$ 100%) revealed a significant decrease in neuronal process length after NS expansion in intact ECCM, and a significant increase in length after expansion in OGD-ECCM (E, * $p \leq 0.01$, + $p \leq 0.05$ vs. controls). F–I: Nestin expression after 4 days of differentiation was low in unconditioned (Uncond) media controls and present in astrocyte-like cells (F), while those from 3T3CCM displayed more varied morphologies (G). NS expanded in intact ECCM and then differentiated for 4 days contained strong nestin expression in radial glial-like cells with long processes (H, arrowheads). NS that were expanded in OGD-ECCM also showed strong nestin-immunoreactivity (I, arrows) and many cells with long radial glia-like processes (I, arrowheads). NS differentiated for 7 days after expansion in control media contained mostly large, flat GFAP+ astrocytes (arrows in J, K), while those from ECCM-expanded NS (L, arrowheads) showed a marked radial glial-like morphology. GFAP+ cells from OGD-ECCM expanded NS displayed a mixture of morphologies suggestive of type II astrocytes (M, arrows) and radial glial-like cells (M, arrowheads). Scale bars: 100 μ m in A (for A–D, J–M); 50 μ m in F (for F–I).

Figure 6. Media conditioned by OGD-treated endothelial cells alters neuroblast chain migration NS were expanded for 5 days, re-plated in Matrigel with conditioned or control media for chain migration assays, and examined after 24 or 72 hours. NS incubated with intact ECCM collected over 6 or 12 hours in serum-free medium (B, E) appeared similar to controls at 24 hours (A, D). NS plated in OGD-ECCM collected 6 or 12 hours post-OGD displayed increased migration at 24 hours (C, F), with numerous chains extending out from the NS. After 3 days in Matrigel, chains extending from NS cultured with OGD-ECCM contained numerous MAP2^{abc}+ immature neurons (green in G) and few GFAP⁺ astrocytic processes (red in G). The length of the longest chain was measured from the center of each NS and graphed as a percent of the unconditioned control ($CM/unconditioned \times 100\%$). OGD-ECCM-treated NS contained significantly longer chains compared with all other conditions (H, $*p < 0.01$). Scale bars: 200 μm in A (for A–F), 25 μm in G.

Table 1

Summary of Neuronal Branching Analyses from SVZ-derived Primary Neurospheres expanded in Conditioned Media and Differentiated for 7 days.

Condition	Number of Neurons/Field	Branch Points/Neuron	Secondary Branches/Neuron	Tertiary Branches/Neuron
Unconditioned	24.62 ± 3.26	1.17 ± 0.15	1.59 ± 0.14	0.59 ± 0.19
6 h 3T3CCM	27.56 ± 3.70	1.23 ± 0.15	1.68 ± 0.15	0.55 ± 0.15
6 h ECCM	39.33 ± 8.86	0.87 ± 0.11	1.20 ± 0.12 [*]	0.42 ± 0.10
6 h OGD-ECCM	36.86 ± 6.92	1.64 ± 0.19 [#]	1.93 ± 0.11	1.17 ± 0.24 ^{**}
12 h 3T3CCM	26.40 ± 3.31	1.29 ± 0.23	1.70 ± 0.25	0.53 ± 0.13
12 h ECCM	29.80 ± 5.38	1.22 ± 0.20	1.56 ± 0.25	0.58 ± 0.13
12 hr OGD-ECCM	29.87 ± 7.18	2.35 ± 0.24 ^{***}	2.34 ± 0.11 ^{##}	1.74 ± 0.33 ^{###}

Significant values after post-hoc testing:

^{*} p=0.01 vs. 6 h 3T3CCM, p<0.05 vs. Uncond and p<0.001 vs. 6h OGD;

^{**} p<0.05 vs. 6 h 3T3CCM and p<0.001 vs. 6h ECCM;

^{***} p<0.005 vs. 12 h 3T3CCM and p<0.0005 vs. 12 h ECCM and Uncond;

[#] p<0.005 vs. 6 h ECCM;

^{##} p<0.005 vs. 12 h ECCM and Uncond;

^{###} p<0.001 vs. 12 h 3T3CCM, 12 h ECCM and Uncond.

Table 2

Summary of the effects of endothelial cells on SVZ neural stem cells.

Condition	Timing	Self-Renewal	Migration	Process Outgrowth	Glial Effects
EC + NS (primary ECs)	co-culture	↑	ND	↑ ↓	++
ECCM (primary ECs)	differentiation	ND	ND	↑ ↓	(* ↑ oligos) ++
ECCM (bEND3)	during expansion	↑ ↑	NO CHANGE	↑ ↓	++
OGD-ECCM (bEND3)	during expansion	NO CHANGE	↑ ↑	↑ ↑	++

* Oligodendrocytes (oligos) increased dramatically in number only in 50% diluted ECCM. Other glial effects (+) involved alteration of Nestin+ and GFAP+ cells toward a more radial glia-like morphology.
ND, Not Done.