Homing of Neural Stem Cells From the Venous Compartment Into a Brain Infarct Does Not Involve Conventional Interactions With Vascular Endothelium

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**Abstract**

Human neural stem cells (hNSCs) hold great potential for treatment of a wide variety of neurodegenerative and neurotraumatic conditions. Heretofore, administration has been through intracranial injection or implantation of cells. Because neural stem cells are capable of migrating to the injured brain from the intravascular space, it seemed feasible to administer them intravenously if their ability to circumvent the blood-brain barrier was enhanced. In the present studies, we found that interactions of hNSCs in vitro on the luminal surface of human umbilical vein endothelial cells were enhanced following enforced expression of cutaneous lymphocyte antigen on cell surface moieties by incubation of hNSCs with fucosyltransferase VI and GDP-fucose (fhNSCs). Interestingly, ex vivo fucosylation of hNSCs not only did not improve the cells homing into the brain injured by stroke following intravenous administration but also increased mortality of rats compared with the nonfucosylated hNSC group. Efforts to explain these unexpected findings using a three-dimensional flow chamber device revealed that transmigration of fhNSCs (under conditions of physiological shear stress) mediated by stromal cell-derived factor 1α was significantly decreased compared with controls. Further analysis revealed that hNSCs poorly withstand physiological shear stress, and their ability is further decreased following fucosylation. In addition, fhNSCs demonstrated a higher frequency of cellular aggregate formation as well as a tendency for removal of fucose from the cell surface. In summary, our findings suggest that the behavior of hNSCs in circulation is different from that observed with other cell types and that, at least for stroke, intravenous administration is a suboptimal route, even when the in vitro rolling ability of hNSCs is optimized by enforced fucosylation.

**Introduction**

Human neural stem cells (hNSCs) hold great potential for the treatment of the behavioral and pathophysiological consequences of a broad range of neurological disorders [1]. One critical observation is that hNSCs are often efficacious not only through neural cell replacement [2–4] but also through a neuroprotective effect on endangered host cells and their microenvironment, a phenomenon known as the “chaperone effect” [2, 5]. As such, neural stem cells (NSCs) are able to rescue endangered host neurons by providing trophic support, detoxifying the milieu, mobilizing endogenous progenitors, triggering self-repair mechanisms, exerting anti-inflammatory and antiscarring actions, and promoting angiogenesis [2, 5]. The combination of these beneficial effects attests to the therapeutic potential of NSCs beyond their function as a cell replacement.

Although the therapeutic potential of NSCs is widely accepted, understanding the most efficient method for delivery of NSCs to the diseased site is a major challenge. Heretofore, administration has typically been intracranial by injection or implantation of cells directly into the damaged or penumbral parenchyma or into the cerebral ventricles [6]. The in situ implantation method ensures that the NSCs are in a proximate location from which they can migrate in response to host-generated signals toward the site of injury or disease [2]. This approach, however, has two major limitations. First, in situ implantation of NSCs is a surgical procedure and thus is traumatic and invasive. Second, for multifocal neurological disorders, a single-cell-implantation procedure may not be sufficient, and multiple injections of NSCs into sites in close proximity to each lesion may not be feasible. These limitations have prompted interest in the intravenous route of delivery of NSCs.
NSC administration. Because we and others have demonstrated that NSCs are capable of circumventing the blood-brain barrier (BBB) and migrating to the injured brain from the intravascular space toward intracranial tumors and sites of chronic inflammation, it seemed feasible to administer NSCs intravenously in cases of stroke as well [7, 8]. However, a limitation of an intravenous approach is that NSCs are extremely inefficient (<5% cells) at homing to the injured brain from the venous compartment. This inefficiency could result from a number of factors. The first consideration is that other organ beds (e.g., spleen, liver, lung) see the injected cells on first pass prior to the brain and remove or trap the cells from circulation. This limitation cannot be overcome in a safe noninvasive manner; a more dangerous intrajugular route would likely be required, raising the risk of increasing intracranial pressure through obstruction to venous outflow and potentially exacerbating an ischemic lesion. In addition, this route would not address another fundamental contributor to poor NSC transmigration through the vascular endothelium: We have previously demonstrated that hNSCs interact poorly with endothelial cells (ECs) under conditions of physiological flow in vitro [9], and this could contribute to their inefficient homing in vivo.

Homing of NSCs to tissues depends on a number of reciprocal interactions between circulating NSCs and ECs. Molecular mechanisms that underlie homing, such as selectin-mediated rolling, integrin-mediated adhesion, and chemokine-directed migration, can be manipulated ex vivo to increase the homing efficiency of cells and subsequently improve the therapeutic potential of cell-based therapies. In the present study, we focused on the mechanism that controls rolling, the first step of the homing cascade, with the goal of enhancing homing of hNSCs injected intravenously at a site distal to the brain (i.e., the tail vein) in a rodent model of a prototypical traumatic brain injury, adult stroke. In other words, we hoped to enhance the ability to circumvent the BBB by altering the antigens on their surface.

We found that the molecules mediating rolling are defective in hNSCs. Specifically, we found that glycoproteins expressed on the cell surface of hNSCs are not fucosylated and hence are unable to successfully interact with selectins. Incubation of hNSCs with fucosyltransferase VI (FT-VI) and its substrate GDP-fucose enforced transient expression of cutaneous lymphocyte antigen (CLA) on cell surface moieties and led to improved rolling and subsequent adhesion of fucosylated hNSCs (fhNSCs) to the luminal surface of ECs in vitro under conditions of physiological shear stress. This process had been postulated to increase hematopoietic stem cell (HSC) and mesenchymal stem cell (MSC) homing in vivo [10, 11]. Human NSCs administered via tail vein in adult rats with experimental stroke (permanent middle cerebral artery occlusion [MCAO]) did migrate into the damaged brain, as described previously [12]. However, in contrast to the situation with HSCs and MSCs, enforced ex vivo fucosylation of hNSCs did not improve their homing into the infarcted brain following intravenous administration via tail vein and did not enhance functional recovery of the animals. Indeed, it resulted in increased mortality of rats compared with control groups. Sorting out the mechanism underlying this unexpected finding should prove extremely instructive to the field of translational stem cell biology. Efforts to explain these unexpected findings relied on the use of a novel three-dimensional (3D) flow chamber device that demonstrated that hNSCs possess a low resistance to physiological shear stress, and that is further decreased by enforced fucosylation, resulting in diminished fhNSC survival. In addition, transmigration of fhNSCs (under conditions of physiological shear stress) as mediated by a stromal cell-derived factor 1α (SDF-1α) gradient, a prominent mediator of hNSC pathotropism in stroke and other neuropathological conditions, was significantly reduced compared with control. In addition, fhNSCs demonstrated a higher frequency of cellular aggregate formation as well as a tendency for removal of fucose from the cell surface. Together, our findings suggest that the behavior of hNSCs in the venous compartment—not a natural niche for such cells—is different from that observed with stem cell types with normal life cycles that do normally include release to such a niche and that, at least for stroke, intravascular administration is a suboptimal route, even when hNSC membrane properties are optimized. Furthermore, damage to the BBB from stroke may be different from that mediated by other neoplastic or neurodegenerative processes.

**MATERIALS AND METHODS**

**Cells**

The fetal-derived hNSCs used in this study are phenotypically and karyotypically stable (46XX) and have been characterized and tested in numerous prior studies over the past 13 years [2, 3, 5, 9, 13]. They are identical to hNSCs that have been used in clinical trials and cluster genomically not only with these hNSCs but also with cells obtained from the human fetal ventricular zone. Cryobanked hNSCs were cultured in chemically defined, serum-free medium, as described [14]. In brief, hNSCs were thawed by dilution into seven volumes of a serum-free culture medium consisting of Neurobasal (Life Technologies, Rockville, MD, http://www.lifetech.com) supplemented with vitamin A-free B27 (Life Technologies) and containing 5 μM l-glutamine or GlutaMax (Life Technologies), 20 μg/ml basic fibroblast growth factor, 10 μg/ml leukemia inhibitory factor, and 8 μg/ml heparin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Cells were collected by centrifugation at 200 relative centrifugal force for 4 minutes, and the pellet was resuspended in 1.0 ml of serum-free culture medium. Viable cells were counted in a hemocytometer using trypan blue exclusion, and the cell count was adjusted to 10^6 hNSCs per cm² of culture vessel surface area. NSCs were propagated in T25 culture flasks (Corning Life Sciences, Acton, MA, http://www.corning.com/lifesciences). Cells were fed every third day with growth factor-supplemented serum-free medium and passaged at ~80% confluence by dissociation with Accutase. NSCs at passage 11 were used in this study. Simultaneous identification of live and dead cells was performed using trypan blue staining. Viability of cells used in all experiments was 95% or greater. To test the static adhesion of hNSCs, Petri dishes were coated with 5 μg/cm² laminin (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com) for 1 hour at room temperature, and hNSCs (6 x 10^5 cells per square centimeter) were placed in these dishes and cultured for 24 hours. Human umbilical vein ECs (HUVECs) were obtained from Lonza (Walkersville, MD, http://www.lonza.com), and human brain-derived microvascular ECs (HBMECs) were obtained from ScienCell (San Diego, CA, http://www.sciencellonline.com). HUVECs and brain-derived ECs (BDECs) were expanded using EC culture kits (Lonza, ScienCell). To ensure phenotypic stability of cells, only ECs having undergone fewer than five passages in culture were used for the in vitro adhesion assays. Human CD34+ cells from umbilical cord blood were from StemCell Technologies (Vancouver, BC, Canada, 230 Neural Stem Cell Homing ©AlphaMed Press 2014
and Zen-Bio (Research Triangle Park, NC, http://www.zen-bio.com). After thawing, cells were washed and kept on ice until use. Human MSCs from bone marrow were from Lonza. MSCs were cultured in minimum essential medium with L-glutamine supplemented with fetal bovine serum premium select (16.5%) and penicillin/streptomycin (100 U/ml and 100 μg/ml), as described previously [15].

Animals

All animal experiments were conducted according to NIH guidelines and in agreement with the Sanford-Burnham Medical Research Institute policy on animal use and were approved by the institutional animal care and use committee. Rats (2–3 months old, Sprague-Dawley females, 210–280 g; Harlan, Indianapolis, IN, http://www.harlan.com) were used to model stroke in vivo.

Permanent MCAO Animal Model

The protocol previously published by Longa et al. [16] has been modified and used in this study (Fig. 1A). Before the surgery, a 5-cm monofilament was created by coating the tip of a 4-0 surgical nylon suture with silicone for a bulbous tip with a diameter of 0.3 mm (gift from Suzanne Marcantonio, Loma Linda University Medical Center, Loma Linda, CA) [17]. Briefly, adult female Sprague-Dawley rats (weight: 210–280 g; Harlan) were anesthetized with 3% isoflurane in 30% oxygen and 70% nitrous oxide, using an anesthetic vaporizer and flowmeter. The rats were then moved to a heating pad (Fisher Scientific International, Hampton, NH, http://www.fisherscientific.com) maintaining their body temperature at 37°C. A 25-mm surgical midline incision was made to expose the right common carotid, external carotid, and internal carotid arteries. Using a sterile cotton-tipped applicator, the carotids were dissected free, and selected arteries feeding into the external and internal carotid were ligated with a 5-0 silk suture (Braintree Scientific, Braintree, MA, http://www.braintreesci.com). Thereafter, a small puncture was made in the external carotid artery stump, and the monofilament was advanced from the lumen of the external carotid artery into the internal carotid artery beyond the bifurcation of the common carotid artery to block the origin of the middle cerebral artery (MCA). The suture was left in place for a permanent occlusion (Fig. 1B). The wounds were closed, and lidocaine cream was applied to the incision area. After the surgery (45 minutes per rat), the rats were allowed to recover on a heating pad with access to food and water ad libitum. The rats were euthanized with an overdose of isoflurane and perfused with 4% paraformaldehyde. The use of animals in this study was approved by the institutional animal care and use committee of Sanford-Burnham Medical Research Institute, and all procedures were carried out in accordance with institutional and NIH guidelines.

Cell Transplantation

Twenty-four hours after permanent MCAO surgery, each rat was restrained in a DepcapiCone (Braintree Scientific) with the tail end

Figure 1. Illustration of the rat MCA occlusion (MCAO). (A): Schematic representation of the surgical procedure. A suture was snaked through the ECA through the ICA to prohibit blood flow to the MCA. (B): Blood flow was occluded to one hemisphere of the brain as indicated in gray. (C): Infarct area detected after MCAO surgery compared with control hemisphere. Animals with permanent MCAO injury exhibit infarction in the left hemisphere compared with the right. Brain sections were stained with 0.001% crystal violet and scanned on a printer. Abbreviations: CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; MCA, middle cerebral artery; PA, pterygopalatine artery; PCA, posterior cerebral artery.
sealed with a large clip and a slit cut open allowing for access to the tail. Cyclosporin (100 mg/kg) was subcutaneously injected before cell transplantation. Thereafter, the tail was dipped into warm water and rubbed until two tail veins were visible to the naked eye. An insulin needle attached to a syringe was used to inject hNSCs. Subsequently, $1 \times 10^6$ sham-treated hNSCs (shhNSCs) or fucosylated hNSCs (fhNSCs) were injected into rat tail veins. Rats were sacrificed 24 hours after hNSC injection; brains were collected and analyzed using immunohistochemistry.

### Fucosylation Reaction

Human NSCs ($10^6$ cells) were treated with concentrations of FT-VI ranging from 1 to 100 $\mu$g/ml and 1 mM of substrate, GDP-fucose, in Hank’s Balanced Salt Solution containing 1% human serum albumin for 30 minutes to 2 hours. For control samples, shhNSCs were incubated in the same buffer in the absence of FT-VI. Where indicated, 30 $\mu$g/ml fucosyltransferase VII was added into the fucosylation mixture and omitted in control samples. The efficiency of $\alpha 1$-3-fucosylation of cell-surface glycans was determined by fluorescence-activated cell sorting (FACS) analysis of bound HECA-452 monoclonal antibody, which recognizes CLA determinants on cell surface glycoproteins.

### Immunofluorescence

Brains were isolated from rats perfused with 4% paraformaldehyde and sectioned on a vibratome at 1 mm in 1 phosphate buffered saline (PBS). A total of 40–60 slides from each brain were prepared for analysis. Sections were permeabilized in 0.1% Triton $\times 100$ for 30 minutes and blocked in 5% bovine albumin serum overnight at 4°C. To detect transplanted hNSCs, the sections were incubated with a 1:500 dilution of mouse antihuman antimitochondria antibody (MAB1273; Millipore, Billerica, MA, http://www.millipore.com) overnight at 4°C. The next day, the sections were washed three times in 1 PBS and incubated in donkey anti-mouse AlexaFluor-488-conjugated secondary antibody (Life Technologies) for 30 minutes at room temperature. Human ECs were grown on the membrane. HUVECs or adherent cells per field, representing the mean ± SD from five capillaries.

### Crystal Violet Staining

Brains were sectioned at 100 mm on a vibratome in 1 PBS. Brain sections were placed in 0.001% crystal violet in distilled water (filtered at 0.45 mm before use) at room temperature for 30 minutes. Sections were washed three times in 1 PBS and then mounted on glass slides with a glass coverslip (Fig. 1C).

### In Vitro Microcapillary Flow Assay

Rolling and adhesion of cells under physiological shear stress was assessed in vitro using microcapillary tubes coated with a monolayer of ECs. Briefly, glass capillaries (Fisher Scientific) were coated with 2% 3-aminopropyltriethoxysilane in acetone (Sigma-Aldrich), washed twice with PBS, dried, and sterilized. Thereafter, the capillaries were coated with 5 mg/ml gelatin type B (Sigma-Aldrich) for 30 minutes at 37°C. Human ECs were grown in the glass capillaries until 100% confluent. Defined levels of flow (wall shear stress) were applied to the capillaries by perfusing warm media (RPMI containing 0.75 mM Ca$^{2+}$ and Mg$^{2+}$ and 0.2% human serum albumin) through a constant infusion syringe pump (Harvard Apparatus, Holliston, MA, http://www.harvardapparatus.com). The capillaries were then perfused with 10 ml of cell suspension (1 $\times 10^5$ cells per milliliter) at various levels of shear stress. At least five EC-coated capillaries were run in each experimental group ($n$ = 5). The interactions of the injected cells with the endothelial layer were observed in the central sector of each capillary using an inverted phase contrast microscope, and the images were recorded. Rolling cells demonstrated multiple discrete interruptions and flowed slowly, whereas adherent cells remained stationary at a given point for extended periods of time (>30 seconds). All results are expressed as the number of rolling or adherent cells per field, representing the mean ± SD from five capillaries.

### 3D Flow Chamber Assay

The device was custom made by C.B.S. Scientific Company Inc. (San Diego, CA, http://www.cbsscientific.com) and used as described previously [18]. Briefly, it consists of upper compartments subjected to physiological flow underneath, where there are static compartments separated by a membrane (5-μm pore size) with the endothelial monolayer. All parts of the device were sterilized by γ-irradiation. The membranes that separate the flow compartment from the static compartment were precoated with 5 μg/cm$^2$ collagen for 60 minutes at room temperature. Human ECs were grown on the membrane. HUVECs were seeded at a concentration of 3 $\times 10^5$ cells per insert, and BDEC were seeded at 5 $\times 10^4$ cells per insert and cultured overnight. The dynamic of cell growth was monitored under an inverted microscope. When ECs were 100% confluent, the wells of the low compartment were filled either with plain culture media (negative control) or with media supplemented with SDF-1α (20 ng/ml), and the inserts were transported into the wells of the bottom block. The top block was connected to the bottom block by screws and attached to sterile catheters connected to a gas exchange unit through a peristaltic pump. Thereafter, by using the negative pressure created by the pump, the chamber was filled by placing the inlet catheter in the 15-ml tube containing culture media. NSCs resuspended in media were drawn into the chamber by suction. Next, the inlet was connected to the outlet catheter, and the defined level of shear stress was applied by using a regulatory switch on the pump. We used a 0.8 dyne/cm$^2$ wall shear stress level because it closely resembles flow conditions in the microvasculature [19]. The chamber and the pump were then placed in the cell culture incubator, and the circulating cells were allowed to interact with the endothelial monolayer at 37°C. The cells which remained in circulation were collected through the outlet, the chamber was disassembled, and the inserts removed. The transmigrated cells were harvested from each well of the lower compartment and counted under the microscope with a hemocytometer counting the total cell number and the percentage of dead cells, as detected by trypan blue uptake.
Flow Cytometry

Detection of CLA on cells was determined with a standard FACS protocol using HECA-452 antibodies (BD Pharmingen, San Diego, CA, http://www.bd.com). FITC-conjugated isotype control antibodies were from SouthernBiotech (Birmingham, AL, http://www.southernbiotech.com). The presence of VCAM on the HUVEC cell surface was detected by mouse anti-human CD106 (VCAM-1, clone #1.G11B1) antibody (SouthernBiotech). Antibodies specific to P-selectin (FITC-conjugated mouse anti-human, clone #AC1.2) and L-selectin (FITC-conjugated mouse anti-human, clone #DREG-56) are from BD Pharmingen. E-selectin antibody (FITC-conjugated, mouse anti-human, clone #CL2) was from eBioscience Inc. (San Diego, CA, http://www.ebioscience.com). To detect integrin expression, anti-CD49a-PE (#328303), anti-CD49b (#3134304), anti-CD49c-PE (#343803), anti-CD49e-FITC (#328007), anti-CD49f-FITC (#313605), anti-α v-FITC (#327907), and anti-CD29-PE (#303003) from BioLegend (San Diego, CA, http://www.biolegend.com/) and anti-CD49d-PE (#580972) and anti-CD44-PE (#553133) from BD Pharmingen were used. Anti-CXCR4 antibody was from the NIH AIDS Reagent Program (clone 44717). Fluorescence intensity was analyzed on a FACScalibur (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) according to standard procedures.

Confocal Microscopy

Cultured hNSCs were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, http://www.invitrogen.com/microscopy) in PBS (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) for 30 minutes. After washing and blocking with 2% fetal calf serum for 2 hours at room temperature, the cells were stained with CLA-specific PE-conjugated antibody for 30 minutes at 4°C. Negative controls were incubated with isotype-matched FITC-conjugated IgG. After washing and staining nuclei with DAPI (Sigma-Aldrich) for 10 minutes, the cells were washed and covered with a drop of AntiFade (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Images were taken on an Olympus Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan, http://www.olympus-global.com).

Statistical Analysis

Results were organized into a database and statistically analyzed using Excel (Microsoft, Redmond, WA, http://www.microsoft.com). The difference in the mean changes in the observed parameters in different groups and the SD of the change were calculated. Two-sided t test was used to verify statistical relevance of the results at a significance level of \( p = .05 \).

**RESULTS**

**Human NSCs Lack the Expression of CLA on Their Cell Surface**

We have previously reported that rolling of hNSCs is undertaken by a subset of integrins [9] instead of a selectin-mediated pathway, which mediates rolling of leukocytes. Because physiologically relevant selectin ligands must be α1-3 fucosylated to form terminal glycan determinants, we tested the expression of CLA on hNSCs. FACS analysis demonstrated a lack of CLA expression on the cell surface of hNSCs (Fig. 2A). Human CD34+ cells (representing human HSCs [hHSCs]) and human MSCs (hMSCs) were used as positive and negative controls, respectively [10, 20]. This finding suggests that selectin ligands are not involved in hNSC migration because of their lack of CLA expression. Thus, enforced expression of CLA should allow the engagement of a selectin-mediated pathway and subsequently improved interactions between hNSCs and ECs.

Conditions that had been previously shown to fucosylate HSCs and MSCs resulted in negative results in the hNSCs. Specifically, incubation of hNSCs with 10 μg/ml FT-VI and 1 mM GDP-fucose for 30 minutes at room temperature did not induce CLA expression. In contrast, incubation of hHSCs in similar fucosylation conditions increased the expression of CLA from 40% to 100%. The intensity of CLA staining was increased from 51 in control nontreated hHSCs to 228 in fucosylated hHSCs. Fucosylation of hMSCs using similar conditions resulted in increased CLA expression in 100% of cells. In addition, the intensity of CLA staining increased from 9 in control nontreated hMSCs to 74 in fucosylated hMSCs (Fig. 2B). For hNSCs, optimization studies revealed that to induce the expression of CLA, these cells had to be incubated with 40 μg/ml FT-VI and 1 mM GDP-fucose for 1 hour at room temperature (supplemental online Fig. 1). This protocol increased the intensity of CLA staining from 9 in control nontreated hNSCs and in shNSCs to 167 in fhNSCs (Fig. 2B).

To assess the physiological relevance of the enforced fucosylation, it is important to define the time frame within which fucosylated cells maintain CLA expression. Following the optimized protocol for fucosylation, hNSCs were kept at +4°C for 5 hours, followed by immunostaining with antibodies specific to CLA. FACS analysis of samples demonstrated that within 5 hours, expression of CLA on hNSCs was not significantly changed (supplemental online Fig. 2A).

In addition, it was tested whether the morphology of hNSCs and their ability to adhere to laminin was affected as a result of fucosylation. Following optimized FT-VI treatment, the ability of fhNSCs to adhere to laminin was not changed and was found to be similar to shNSCs (supplemental online Fig. 2B). No apparent morphological changes were observed.

**Fucosylation Improves Rolling of hNSCs on the Endothelial Monolayer**

Because fucosylation of cell surface glycoproteins expressed on migrating cells improves their rolling under conditions of physiological shear stress [10, 11, 21], we tested the effect of enforced fucosylation on hNSC rolling using a microcapillary assay. Fucosylated hHSCs and hMSCs were used as positive controls. Sham-treated and fucosylated cells were passed through microcapillaries with a monolayer of activated HUVECs under physiological shear stress of 0.8 dyne/mm². Interactions of cells with the endothelial monolayer were recorded and analyzed by counting the number of rolling and adherent cells per microscopic field of observation.

Our results show that despite the lack of CLA, the efficiency of shNSC rolling on EC was twofold higher compared with control hHSCs and hMSCs (supplemental online Fig. 3A), confirming our previous findings demonstrating an involvement of other adhesion molecules such as integrins in mediating hNSC rolling [9]. Similarly, adhesion of shNSCs to EC under 0.8 dyne/mm² wall shear stress was also higher than hHSCs but was not different from hMSCs (supplemental online Fig. 3B).
As expected, fucosylation of hNSCs significantly increased the number of both rolling and adherent fhNSCs to HUVECs (Fig. 3A). This is in line with previously reported observations demonstrating increased rolling and adhesion of hHSCs and hMSCs in vitro following fucosylation [10, 11, 21] (Fig. 3B, 3C), which also correlated with their improved engraftment in vivo [10, 11]. This observation led us to suggest that the interactions of circulating fhNSCs with the vasculature following intravenous injection into animals could be improved, which may translate into improved homing.

The Effect of Fucosylation on hNSC Migration In Vivo
Stroke is one pathological condition in which administration of NSCs may mitigate further damage to brain functions. Specifically, transplantation of NSCs into ischemic rodent models correlates with improved histological and functional outcome [22]. Because the effective interaction of intravenously administered NSCs with the vasculature is required for their homing into the sites of brain injury, we tested whether fucosylation was a viable method to enhance hNSC rolling and, subsequently, homing.

To model human ischemia, we replicated stroke in a rat via MCAO surgery to block blood flow to the left hemisphere (Fig. 1). Twenty-four hours after surgery, shNSCs or fhNSCs were injected into the tail vein of the rats, and then the brains were examined for the presence of hNSCs. Both shNSCs and fhNSCs were detected in the injured left hemisphere of the brain (Fig. 4A). Quantitative evaluation of the number of migrated hNSCs demonstrated that fucosylation did not significantly increase the number of fhNSCs that migrated into the left hemisphere of the brain compared with shNSCs that migrated to the left hemisphere (p = .25) (Fig. 4B).

Interestingly, fhNSCs were detected in the noninjured right hemisphere of the brain, whereas shNSCs were not detectable. Quantitative analysis showed that there was a statistically significant difference between the number of shNSCs that migrated to the right hemisphere versus the left hemisphere (p = .0001). After fucosylation, the number of fhNSCs that migrated to the left hemisphere was also higher compared with the right hemisphere; however, this difference was not statistically significant (p = .17) (Fig. 4B). Thus, systemically administered hNSCs preferentially migrated to the injured hemisphere independent of surface fucosylation.

Surprisingly, increased mortality was noticed in rats injected with fhNSCs. Control rats after MCAO surgery that did not receive intravenous infusion of hNSCs demonstrated 100% survival (n = 6). Rats that received shNSCs had a 30% death rate (n = 6). Rats that received fhNSCs had an 83% death rate (n = 6). This prompted additional more detailed evaluation of brain sections. We found that after fucosylation, fhNSCs showed increased formation of cellular aggregates (Fig. 4C), which may have caused the deaths of the animals.

Figure 2. The expression of CLA on adult stem cells. (A): The expression of CLA on control human CD34+ cells from umbilical cord blood (referred to as human HSCs [hHSCs]), on human MSCs [hMSCs] from bone marrow, and on human NSCs [hNSCs] from fetal forebrain was tested by fluorescence-activated cell sorting. (B): hHSCs and hMSCs were fucosylated using 10 μg/ml FT-VI and a 30-min incubation time. nNSCs were fucosylated by treatment with 40 μg/ml FT-VI for 1 hour. Representative histograms show CLA expression. Abbreviations: CLA, cutaneous lymphocyte antigen; FT-VI, fucosyltransferase VI; HSC, hematopoietic stem cell; MSC, mesenchymal stem cell; NSC, neural stem cell.
Fucosylation Negatively Affects SDF-Mediated Transmigration of hNSCs Under Conditions of Physiological Shear Stress

The unexpected results from the in vivo experiments prompted additional in vitro tests to provide a better understanding of changes in hNSC biology following fucosylation. For these studies, we used a 3D flow chamber device to mimic flow conditions in the brain. Because species-specific differences in rat versus human endothelium may provide a reason for the apparent failure of fhNSC homing to the rat brain, we used HBMECs in this test. HBMECs were grown on the inserts until 100% confluent, treated with tumor necrosis factor-α, and placed in wells containing plain media or media supplemented with SDF-1α. Sham-treated hNSCs and fhNSCs were allowed to circulate in the 3D device for 24 hours. Transmigration of shhNSCs across the HBMEC barrier was enhanced in the presence of an SDF-1α gradient. By contrast, transmigration of fhNSCs to the SDF-1α gradient was significantly less (three- to fourfold) than in sham-treated cells. Although the expression of CXCR4 in fucosylated hNSCs was not changed (not shown), the results suggest that mechanisms underlying SDF-1α-mediated chemotactic motility were impaired when cells were fucosylated prior to testing (Fig. 5A).

Because the biology of ECs, including their cell surface, depends on their origin, we compared the efficiency of adhesive interactions of hNSCs with activated HUVECs versus HBMECs. The number of hNSCs that roll on HUVECs was significantly higher compared with the number of hNSCs that roll on HBMECs, whereas the adhesion of hNSCs to HUVECs and to human BDECs was similar (supplemental online Fig. 3C).

Figure 3. Effect of fucosylation on human stem cell interactions with an endothelial monolayer. Rolling and adhesion of shNSCs and fhNSCs (A), hHSCs (B), and hMSCs (C) on activated human umbilical vein endothelial cells under conditions of various shear stresses were studied using a microcapillary assay (five microcapillaries per group). Numbers of rolling and adherent hNSCs, hHSCs, and hMSCs per microscopic field of observation located in the center of each capillary are shown. Results of one representative experiment of three similar experiments are shown as mean ± SD of triplicates for cells at a concentration of 10⁴ cells per milliliter. A statistically significant difference between sham and fucosylated cells was noted in all tests (p < .05). Abbreviations: fhNSC, fucosylated human neural stem cell; hHSC, human hematopoietic stem cell; hMSC, human mesenchymal stem cell; hNSC, human neural stem cell; shNSC, sham-treated human neural stem cell.
Human NSCs Have Low Resistance to Physiological Shear Stress

After 24 hours, hNSCs remaining in the circulation in the 3D device were collected from rats with stroke injected with sham-treated hNSCs or fucosylated hNSCs. Presence of hNSCs in right (healthy) and left (post-stroke) hemisphere was tested by detecting human mitochondria (green). Nuclei were stained with 4’,6-diamidino-2-phenylindole (blue). (B): Quantitative evaluation of the numbers of hNSCs migrated in both hemispheres is shown. The cells were counted manually by microscopically examining randomly selected fields in representative slides (n = 9). (C): A representative image shows the presence of microaggregates in brains of rats injected with fucosylated hNSCs. Abbreviations: FT-VI/VII, fucosyltransferase VI and VII; hNSC, neural stem cell.

To investigate whether the increased cell death was caused by shear stress, shNSCs and fhNSCs were placed into static culture dishes. Survival of hNSCs was tested by both propidium iodide incorporation and trypan blue exclusion. We found that the exposure of hNSCs to fucosylation buffer reagents for 1 hour at room temperature induces changes leading to cell death, which gradually increased over time (Fig. 6A). Notably, the percentage of dead cells is higher in the fucosylated cell population compared with sham-treated controls at all time points of incubation. The largest difference between shNSCs and fhNSCs was detected during the first 2–4 hours in the culture dishes (Fig. 6A). Interestingly, the fold change in the number of dead cells between shNSCs and fhNSCs 24 hours after the fucosylation reaction (Fig. 6B) was similar in static conditions compared with conditions of shear stress and suggests that the enforced fucosylation of cell surface glycoproteins may not be favorable to hNSC wellbeing. However, overall cell death was significantly lower under static conditions, suggesting that the ability of hNSCs to tolerate shear stress is low.

Although it could be postulated that permanent fucosylation and permanent change to the hNSC cell membrane led to these adverse results, it is critical to point out that the fucosylation was transient—long enough to enhance interactions with vascular selectins but not long enough to alter the hNSCs’ ability to interact with other instructive ligands in the extravascular central nervous system (CNS) injury niche. Specifically, we observed a decrease in the expression of CLA by ~2 hours after fucosylation, an ideal time...
frame for administration of the hNSCs for a therapeutic effect. Immunocytochemistry demonstrated that CLA expression was intense on fhNSCs after 1 hour and largely disappeared from the surface of fhNSCs by 40 hours (Fig. 7A). This finding was confirmed by FACS analysis, which demonstrated both a decreased intensity of staining (Fig. 7C) and a decreased percentage of hNSCs expressing CLA (Fig. 7B). These results suggest that hNSCs either remove the added fucose or rapidly shed the newly fucosylated moiety from their cell surface.

**DISCUSSION**

Although the therapeutic potential of stem cells is becoming a well-established concept, the optimal route for their administration remains in question. In clinical settings, there is a choice between two approaches: direct local or intralesional implantation versus systemic intravascular, typically intravenous, administration. Direct injection of NSCs regionally or locally into the brain is feasible; however, it is invasive and requires a sophisticated surgical approach that limits broad applicability and could disrupt the highly complex and delicate structure of the local regulatory microenvironment (i.e., the niche), causing additional traumatic injury and inflammation. Another limitation of the local administration of NSCs is the multifocality of many disorders, particularly those of a chronic inflammatory nature (e.g., multiple sclerosis). Intravenous administration of NSCs might address this challenge; however, this approach, in itself, is limited by the difficulty of ensuring that sufficient numbers of NSCs reach the damaged areas, not only because of removal of cells from the circulation by more proximal organs (e.g., spleen, liver, lung, bone marrow) but also because of ineffective NSC exit from the vascular compartment and homing. In this study, we have demonstrated for the first time that hNSCs possess a naturally occurring lack of CLA expression on the cell surface, with the result that the hNSCs interact poorly with vascular selectins. Enforced ex vivo fucosylation of hNSCs reversed this natural deficiency and improved rolling of hNSCs on the luminal surface of ECs under physiological flow in a conventional microcapillary assay. Surprisingly, this did not improve the ability of fhNSCs to migrate into the brain injured by stroke. Additional studies to understand the mechanism underlying these observations using a novel 3D flow chamber device demonstrated that hNSCs possess a low ability to sustain shear stress and that this is further decreased following fucosylation. Consequently, fulfilling the promise of hNSC-based tissue repair and protection critically depends on better understanding of the basic biology of hNSC-related migration and pathotropism.
blood. Xia et al. reported enhanced adhesion and binding of functional significance of fucosylated hHSCs from umbilical cord respectively, controversial results have been obtained with regard to the homing of fucosylated hMSCs to bone marrow was significantly increased [10]. Alternatively, when fucosylation deficiency, similar to that described in this paper, was corrected in hMSCs, homing of fucosylated hMSCs, which is based on studies on leukocyte migration, is that rolling of circulating cells on ECs is cooperatively mediated by P- and E-selectins that are expressed on activated ECs [23–25]. Notably, physiologically relevant selectin ligands must be α1-3 fucosylated to form terminal glycan determinants, CLA [23–27], present on cell surface moieties such as the P-selectin glycoprotein ligand and CD44. Because hNSCs rolled more efficiently compared with hHSCs but 40% of hHSCs express CLA on the cell surface, it was surprising that no CLA was detected on hNSCs. Furthermore, ex vivo fucosylation conditions described for hHSCs and hMSCs [10, 11] were suboptimal for hNSCs, suggesting the presence of endogenous mechanisms preventing fucosylation or cleaving fucose or fucosylated glycoproteins from the cell surface. This is in line with our previously published observations that rolling of hNSCs is mediated by a molecular axis other than selectins and their ligands [9].

In line with previously published observations of the effect of fucosylation on rolling of hHSCs and hMSCs, we found that enforced expression of CLA using an optimized fucosylation protocol improved the ability of hNSCs to roll and adhere to activated HUVECs under conditions of physiological shear stress. As expected, the enforced expression of CLA in hHSCs did not influence the expression of integrins (αv, α1-6, and β1), VCAM1, CXCR4, and CD44 proteins on the cell surface of fhNSCs (not shown).

Because ex vivo fucosylation of stem cells enhances their interactions with ECs in vitro, fucosylation of hNSCs before tail vein injection was considered as an attractive approach to enhance their homing in vivo to the cerebral infarct. Interestingly, when fucosylation deficiency, similar to that described in this paper in hNSCs, was corrected in hNSCs, homing of fucosylated hMSCs to bone marrow was significantly increased [10]. Alternatively, controversial results have been obtained with regard to the functional significance of fucosylated hHSCs from umbilical cord blood. Xia et al. reported enhanced adhesion and binding of fucosylated CD34+ cells that translated to enhanced homing to bone marrow of sublethally irradiated mice and subsequent improved engraftment [11]. In contrast, using in vivo experiments, Hidalgo et al. reported that ex vivo fucosylation of selectin ligands on human umbilical cord blood CD34+ cells enhanced initial interactions with the bone marrow vasculature in vitro, but this did not lead to their enhanced homing [21]. These results are consistent with our present studies in the CNS, where we tested the effect of ex vivo fucosylation on migration of hNSCs into the ischemic area induced by stroke in the brain. As expected, the migration of hNSCs into the injured part of the brain was significantly higher compared with the noninjured area in the same brain; however, we did not observe a significant difference between the homing ability of control shNSCs versus fhNSCs. Indeed, as noted below, we saw increased mortality. One possible explanation is that the microvascular ECs in rat brain possess species-specific differences that cause ineffective interactions of circulating hNSCs with rat brain vasculature. In addition, the changes in the BBB following stroke may be different than those caused by other nontraumatic neurological insults, such as tumors, chronic inflammation (e.g., multiple sclerosis), and neurodegeneration (e.g., amyotrophic lateral sclerosis, Parkinson disease).

To investigate this possibility, we used a novel 3D flow chamber device that allows us to investigate interactions of circulating cells with ECs under conditions of physiological shear stress for a chosen period of time and to quantitatively evaluate extravasation of circulating cells toward a chemokine gradient [18]. To better mimic the blood-brain microvasculature, we used HBMECs to separate the upper under-shear-stress compartment from the lower static compartment containing plain media or SDF-1α-supplemented media. As expected, an SDF-1α gradient enhanced transmigration of control shNSCs circulating in the device across the endothelial barrier. Importantly, extravasation of circulating fhNSCs toward an SDF-1α gradient was significantly lower compared with control shNSCs, although the expression of CXCR4 on fhNSCs was not changed. The response to an SDF-1α gradient by CXCR4-bearing hNSCs has been shown by us and others to be a fundamental mechanism of stem cell pathotropism toward stroke and other neuropathologies [2, 7]. Most important, we found that 60% of hNSCs die when subjected to physiological shear stress before fucosylation.}

![Figure 6.](image)

**Figure 6.** Effect of fucosylation on hNSC survival. Following fucosylation, fucosylated hNSCs were placed back in culture dishes (five per group). Sham-treated hNSCs served as control. Cells were harvested at the indicated time points, and live cells were detected by their exclusion of trypan blue. (A): The percentage of surviving versus dead cells. (B): The fold difference in the death rate of shNSCs versus fhNSCs. Results of one representative experiment of three similar experiments are shown. The percentage of dead hNSCs is higher in the fucosylated population compared with sham-treated controls at all time points. Cell death was lower under static conditions, suggesting that hNSC tolerance of shear stress is also low. Abbreviations: FT-VI/VII, fucosyltransferase VI and VII; hNSC, neural stem cell.

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**Neural Stem Cell Homing**

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shear stress for 24 hours. This finding suggests that, in contrast to leukocytes or ECs that are "designed" to withstand shear stress, hNSCs possess low tolerance to the physiological shear stress observed in the microvasculature. Interestingly, fucosylation of cell surface glycoproteins further reduced the resistance of hNSCs to shear stress and increased the fraction of dead cells to 80%. The increased aggregate formation, similar to that observed in fhNSC samples, has been postulated to be a self-preservation strategy used by cells "sensing" a risk of cell death [28, 29]. However, this increased clumping following fucosylation of hNSCs might explain the increased mortality of infarcted rats receiving fhNSCs, likely resulting from acute vascular obstruction.

Because fucosylation of cell surface glycoproteins in hNSCs decreases the resistance to shear stress and increases the sensitivity to microenvironmental factors that may cause cell death, it is likely that hNSCs possess mechanisms that cleave fucose. Indeed, this property may reinforce the fact that hNSCs, in contrast to hHSCs and mature hematopoietic cells, do not naturally populate the intravascular space as a part of their life cycle.

**CONCLUSION**

There is little doubt that the ability to enhance interactions of hNSCs with ECs specifically in the brain and to guide them across the BBB would have significant clinical implications for the use of intravenously administered hNSCs in neurological disorders. Thus, identification and manipulation of mechanisms to increase their efficiency in rolling as well as adhering to and transmigrating across the BBB may be therapeutically advantageous. Potentially, by increasing the efficiency of binding of hNSCs to brain vascular endothelium and trafficking into the brain, one could reduce the number of hNSCs needed to achieve a therapeutic effect across a broad range of clinical conditions. However, as reflected in results from the present study, it is now apparent that knowledge generated from studies on leukocytes (that have been naturally designed to tolerate high shear stresses inside the vasculature) is not always applicable to other cell types, including stem cells, that normally reside in parenchyma and are not exposed to shear stress under normal conditions (e.g., hNSCs). The present results also highlight the need for additional studies to better understand how to address hNSC survival, extravasation, and migration toward the sites of injury from the intravascular compartment. Perhaps an arterial route may be more effective for the CNS. Therefore, fulfilling the promise of hNSC-based regenerative medicine critically depends on identifying the range of molecules that mediate organ-specific stem cell homing, how they can best be implemented either singly or in combination, and how these pathways can be regulated to render cells more susceptible to shear stress resistance.
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AUTHOR CONTRIBUTIONS

V.G., S.D., W.N., I.S., A.K.W., T.P., and D.W.: performance of experiments, collection and assembly of data; L.M.: provision of study material (FT-VI/VII and GDP-fucose); E.Y.S. and S.K.K.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

REFERENCES

1 Snyder EY, Daley GQ, Goodell M. Taking stock and planning for the next decade: Realistic prospects for stem cell therapies for the nervous system. J Neurosci Res 2004;76:157–168.
2 Imiola J, Raddassi K, Park Ki et al. Directed migration of neuronal stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci USA 2004;101:18117–18122.
3 Lee JP, Jeyakumar M, Gonzalez R et al. Neural stem cells act through multiple mechanisms to benefit mice with neurodegenerative metabolic disease. Nat Med 2007;13:439–447.
4 Park Ki, Lachyankar M, Nissim S et al. Neural stem cells for CNS repair: State of the art and future directions. Adv Exp Med Biol 2002;506:1291–1296.
5 Ourednik J, Ourednik V, Lynch WP et al. Neural stem cells display extensive tropism for pathology in adult brain: Evidence from intracranial gliomas. Proc Natl Acad Sci USA 2000;97:12846–12851.
6 Pluchino S, Quattrini A, Brambilla E et al. Injection of adult neuroprogenitors induces recovery in a chronic model of multiple sclerosis. Nature 2003;422:688–694.
7 Mueller FJ, Soroceanu L, Schraffstatter IU et al. Adhesive interactions between human neural stem cells and inflamed human vascular endothelium are mediated by integrins. Stem Cells 2006;24:2367–2372.
8 Sackstein R, Merzaban JS, Cain DW et al. Ex vivo glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. Nat Med 2008;14:181–187.
9 Xia L, McDaniel JM, Yago T et al. Surface fucosylation of human cord blood cells augments binding to P-selectin and E-selectin and enhances engraftment in bone marrow. Blood 2004;104:3091–3096.
10 Song M, Kim Y, Kim Y et al. MRI tracking of intravenously transplanted human neural stem cells in rat focal ischemia model. Neurosci Res 2009;64:235–239.
11 Redmond DE Jr., Bjugstad KB, Teng YD et al. Behavioral improvement in a primate Parkinson’s model is associated with multiple homeostatic effects of human neural stem cells. Proc Natl Acad Sci USA 2007;104:12175–12180.
12 Wakeman DR, Hofmann MR, Redmond DE Jr et al. Long-term multilayer adherent network (MAN) expansion, maintenance, and characterization, chemical and genetic manipulation, and transplantation of human fetal forebrain neural stem cells. Curr Protoc Stem Cell Biol 2009;9:2D.3.1–2D.3.7.
13 Schraffstatter IU, Discipio RG, Zhao M et al. C3a and C5a are chemotactic factors for human mesenchymal stem cells, which cause prolonged ERK1/2 phosphorylation. J Immunol 2009;182:3827–3836.
14 Longa EZ, Weinstein PR, Carlson S et al. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989;20:84–91.
15 Aspey BS, Cohen S, Patel Y et al. Middle cerebral artery occlusion in the rat: Consistent protocol for a model of stroke. Neuropathol Appl Neurobiol 1998;24:487–497.
16 Goncharova V, Khalaidyonid SK. A novel three-dimensional flow chamber device to study chemokine-directed extravasation of cells circulating under physiological flow conditions. J Vis Exp 2013;e50595.
17 Mazo IB, Gutierrez-Ramos JC, Frenette PS et al. Hematopoietic progenitor cell rolling in bone marrow microvessels: Parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. J Exp Med 1998;188:465–474.
18 Merzaban JS, Burdick MM, Gadhoum SZ et al. Analysis of glycoprotein E-selectin ligands on human and mouse marrow cells enriched for hematopoietic stem/progenitor cells. Blood 2011;118:1774–1783.
19 Hidalgo A, Weiss LA, Frenette PS. Functional selectin ligands mediating human CD34 (+) cell interactions with bone marrow endothelium are enhanced postnatally. J Clin Invest 2002;110:559–569.
20 Bliss TM, Andris RH, Steinberg GK. Optimizing the success of cell transplantation therapy for stroke. Neurobiol Dis 2010;37:275–283.
21 McEver RP. Adhesive interactions of leukocytes, platelets, and the vessel wall during hemostasis and inflammation. Thromb Haemost 2001;86:746–756.
22 McEver RP. Selectins: Lectins that initiate cell adhesion under flow. Curr Opin Cell Biol 2002;14:581–586.
23 Vestweber D, Blanks JE. Mechanisms that regulate the function of the selectins and their ligands. Physiol Rev 1999;79:181–213.
24 Xia L, Sperandio M, Yago T et al. P-selectin glycoprotein ligand-1-deficient mice have impaired leukocyte tethering to E-selectin under flow. J Clin Invest 2002;109:939–950.
25 Yang J, Furie BC, Furie B. The biology of P-selectin glycoprotein ligand-1: Its role as a selectin counterreceptor in leukocyte-endothelial and leukocyte-platelet interaction. Thromb Haemost 1999;81:1–7.
26 Day ML, Zhao X, Vallorosi CJ et al. E-cadherin mediates aggregation-dependent survival of prostate and mammary epithelial cells through the retinoblastoma cell cycle control pathway. J Biol Chem 1999;274:9656–9664.
27 Vallorosi CJ, Day KC, Zhao X et al. Truncation of the beta-catenin binding domain of E-cadherin precedes epithelial apoptosis during prostate and mammary involution. J Biol Chem 2000;275:3328–3334.

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