Intracarotid Injection of Fluorescence Activated Cell-Sorted CD49d-Positive Neural Stem Cells Improves Targeted Cell Delivery and Behavior After Stroke in a Mouse Stroke Model

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Background and Purpose—Intravascular delivery of neural stem cells (NSCs) after stroke has been limited by the low efficiency of transendothelial migration. Vascular cell adhesion molecule-1 is an endothelial adhesion molecule known to be upregulated early after stroke and is responsible for the firm adhesion of inflammatory cells expressing the surface integrin, CD49d. We hypothesize that enriching for NSCs that express CD49d and injecting them into the carotid artery would improve targeted cell delivery to the injured brain.

Methods—Mouse NSCs were analyzed for the expression of CD49d by fluorescence activated cell sorting. A CD49d-enriched (CD49d+/H11001 >95%) and -depleted (CD49d–; <5%) NSC population was obtained by cell sorting. C57/B16 mice underwent left-sided hypoxia–ischemia surgery and were assigned to receive 3×10⁵ CD49d+ NSCs, CD49d– NSCs, or vehicle injection into the left common carotid artery 48 hours after stroke. Behavioral recovery was measured using a rotarod for 2 weeks after cell injection.

Results—Fluorescence activated cell sorting analysis revealed 25% CD49d+ NSCs. In a static adhesion assay, NSCs adhered to vascular cell adhesion molecule-1 in a dose-dependent manner. Significantly more NSCs were found in the cortex, the hippocampus, and the subventricular zone in the ischemic hemisphere in animals receiving CD49d+ NSCs as compared with CD49d– NSCs (P<0.05). Animals treated with CD49d+ cells showed a significantly better behavioral recovery as compared with CD49d– and vehicle-treated animals.

Conclusions—We show that enrichment of NSCs by fluorescence activated cell sorting for the surface integrin, CD49d, and intracarotid delivery promotes cell homing to the area of stroke in mice and improves behavioral recovery.

Key Words: CD49d ■ neural stem cells ■ stroke ■ VCAM-1

Transplanted human neural stem cells (NSCs) have been shown to survive and differentiate in the brain of rats with stroke and to improve behavioral recovery. Recently, the first clinical trials have been carried out in patients with stable and persistent stroke deficits. Many questions remain to be addressed when considering potential clinical applications of stem cell therapy. One of the outstanding questions is the optimal route of delivery. Focal intraparenchymal cell delivery has the disadvantage of being invasive and results in a limited distribution of cells along the lesioned area. A less invasive route is delivery into the intravascular compartment. Recent studies of intravascular delivery of mesenchymal stem cells and NSCs have shown homing of injected stem cells toward the injured central nervous system. One problem of systemic delivery of stem cells is that only a very limited number of injected cells home to the site of injury. It has been suggested that NSCs and immune cells share a similar mechanism for transendothelial migration. Pluchino et al have shown that NSCs express the surface integrin CD49d (also called very late antigen-4) that allows them to adhere to the adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), which is upregulated in the inflammatory response of the brain to injury, including stroke.

We tested whether NSCs enriched for CD49d by fluorescence activated cell sorting (FACS) and injected into the common carotid artery in mice after stroke would show greater homing than CD49d-negative cells and whether there was improved behavioral outcome.

Materials and Methods

Cell Culture
C17.2 NSCs were donated by Dr Evan Y. Snyder (The Burnham Institute, La Jolla, Calif). C17.2 is a clonal, multipotent neural
The cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 5% horse serum (Gibco), and 1% L-glutamine (BRL, Gaithersburg, Md). Medium was changed every other day and cells were passaged once a week. For the in vivo experiments, we prelabeled the cells with bromodeoxyuridine (BrdU). BrdU (10 μmol/L) was added to the cell culture medium for 48 hours followed by a 24-hour chase.

Cell Adhesion Assay
The ability of NSCs to adhere to VCAM-1 was tested in a static cell adhesion assay. Multiswell cell culture plates were coated with laminin (10 μg/mL) as the positive control (BD Bioscience, San Jose, Calif), VCAM-1 (10 μg/mL; R&D; diluted in 0.1 mmol/L phosphate-buffered saline (PBS) to 1:40, 1:100, 1:400) or no coating (negative control) at 4°C overnight. The plates were washed with 0.1 mmol/L PBS and 3% goat serum. NSC cells were labeled with DiI according to the manufacturer’s instructions (CellTracker CM-Dil; Invitrogen, Carlsbad, Calif) before being added to wells. After 30 minutes at 37°C and 5% CO2, the wells were rinsed 3 times with PBS. Adhering fluorescently labeled NSCs were then quantified using a fluorescence plate reader (FlexStation 3 microplate reader; Molecular Devices, Sunnyvale, Calif).

Cell Sorting
Cells were incubated with a rat antimouse CD49d antibody (1:200; GenTex, San Antonio, Texas) or isotype control and positively selected using the StemSep (StemCell Technologies Inc, Vancouver, BC, Canada) magnetic bead system. The positively selected cells were further stained with a polyethylene-conjugated antirat secondary antibody. The labeled cells were double-sorted with a 3-laser FACSSuite (Becton Dickinson, San Jose, Calif). The appropriate forward scatter and side scatter and propidium iodide (10 μg/mL) gating was used to isolate viable cells. After sorting, the purity of CD49d-positive and -negative cells was reanalyzed by flow cytometry. The sorted cells were resuspended in PBS/glucose at a concentration of 6×10⁴ cells/μL and placed on ice until cell injection. A sample of CD49d-positive and -negative cells was reanalyzed before sorting in 4-well chamber slides. After 24 hours, the cells were fixed in paraformaldehyde and stained for glial fibrillary acid protein and nestin (see below). For each condition, 5 fields of view were counted and the percentage of double-positive cells calculated.

Stroke Surgery and Cell Injection
All animal procedures were approved by the Stanford University Administrative Panel on Laboratory Animal Care. The study design is outlined in Figure 1. Stroke was induced using a hypoxia ischemia stroke model.²⁷–²⁹ Briefly, C57Bl6 (n=18) mice underwent unilateral common carotid artery occlusion using an aneurysm clip. The animals were allowed to recover for 2 hours before hypoxia–ischemia. Hypoxia–ischemia was induced by exposure of the animals to 8%O₂ during 20 minutes in temperature-controlled chambers set at 36°C. Reperfusion was performed after hypoxia by removing the aneurysm clip.

Intracarotid cell injection was performed 48 hours after stroke. The common carotid artery was reexposed and 5 μL of single cell suspension (3×10⁵ cells) injected using a 10 μL Hamilton syringe with a 33G needle. Group (A) received CD49d-enriched cells, group (B) received CD49d-depleted cells, and group (C) saline injection. An additional control group of nonstroked animals received stereotaxic (Kopf Instruments) intrastriatal injection of sorted cells (1.2×10⁵ cells) at the following coordinates: from bregma, lateral 1.5 mm, anterior 0.5 mm, depth 2.0 mm.

Behavior
Behavioral assessment was performed on a rotarod (Rotamex-5; Columbus Instruments, Columbus, Ohio). Mice were accommodated and trained on the rotarod with accelerating speed 3 days before stroke surgery. A baseline measurement was acquired the day before stroke. The animals recovered for 2 days after stroke and were tested again before cell or buffer injection. To obtain 3 groups with equal poststroke behavior, the animals were ranked based on their poststroke rotarod results and assigned to one of the 3 groups. All animals were restevaluated at 10 and 17 days after stroke.

Histology and Immunocytochemistry
After completion of the behavior experiments, animals were transcardially perfused with saline followed by 3% paraformaldehyde and processed for immunohistochemistry. Detection of the BrdU-prelabeled cells was done using BrdU immunostaining. Standard immunohistochemical staining techniques were used on free-floating sections or pure free acid fixed adhering cells. Sections or cells were incubated in blocking solution (PBS containing 3% normal serum of the species in which the secondary antibody was raised) for 1 hour at room temperature. Sections were incubated overnight at 4°C in primary antibodies diluted in PBS containing 1% serum. The
following antibodies were used: antinestin (1:1500; R&D Systems), antidoublecortin (DCX, 1:100; Santa Cruz Biotechnology), anti-β-tubulin III (TuJ-1, 1:2000; Covance Research Products, Berkeley, Calif), antiglial fibrillary acid protein (1:800; Advanced ImmunoChemicals, Long Beach, Calif), antilectin (1:25050; Vector Labs), anti-VCAM-1 (1:500; R&D Systems), anti-Iba-1 (1:500; Abcam Inc), and anti-CD68 (1:250; Abcam Inc). After several washes in PBS/1% serum, sections were incubated with secondary antibodies for 1 hour (Alexa Fluor 488 or 568, 1:1000; Molecular Probes; goat–antirabbit Cy3, 1:2000; Jackson ImmunoResearch) followed by multiple washes and nuclear staining with DAPI (1:1000; Invitrogen). Fluorescence-labeled sections were analyzed and double-labeling confirmed using z-stacks acquired on a laser scanning confocal microscope (LSM5; Zeiss). All quantifications were done using Image J (National Institutes of Health software). Nissl-stained sections were used to calculate the stroke volume and the percentage loss of tissue in the stroked hemisphere compared with the contralateral hemisphere. To analyze the inflammatory response, Iba-1 and CD68-positive cells were counted in the penumbral area. For each animal, a minimum of 100 cells was counted and the percentage of double-positive cells determined. Angiogenesis was analyzed using lectin-stained sections. The lectin-positive surface was measured on 3 consecutive sections per animal for all groups. The contralateral hemisphere was used as an internal control. V-CAM-1 and lectin stainings were also performed 48 hours poststroke.

Results

Expression of CD49d and In Vitro Cell Adhesion Assay

FACS results showed that 25% of analyzed NSCs were positive for CD49d (Figure 2A–B).

After demonstrating the presence of CD49d on NSCs, we studied cell adhesion in vitro using a static cell adhesion assay. Laminin (positive control 10 μg/mL), VCAM-1 (10 μg/mL) coated or uncoated (negative control) multwell plates were seeded with DiI-labeled NSCs and subsequently analyzed by a fluorescence plate reader for adhering cells. Laminin and VCAM-1 at a dilution of 1:40 were not significantly different for the adherence of NSCs. At a VCAM-1 dilution of 1:100 and 1:400, there was a significant decrease in cell adhesion as compared with laminin (Figure 2C). Laminin and all VCAM-1-coated wells were significantly more adherent for NSCs than uncoated wells (Figure 2C).

Cell Sorting

Double FACS sorting led to a >95% enriched CD49d-expressing cell population and a <5% depleted cell popula-
tion. Samples from both the positively and negatively sorted cells were plated after the sorting process and immunohistochemistry was done for CD49d (Figure 3A–D). There was no staining in the depleted NSC population (Figure 3D), whereas we found that most of the positively selected cells expressed CD49d in a cluster-like pattern (Figure 3B). Cell viability after sorting was >90% as determined by the trypan blue dye exclusion test. To confirm that the cells retained a progenitor-like phenotype after FACS, samples of cells were stained for nestin (Figure 3E) and glial fibrillary acid protein (Figure 3F) after the sorting process. No difference in the expression profiles was found between CD49d-positive and -negative sorted cells (Figure 3G).

Transendothelial Migration of CD49d-Sorted Neural Stem Cells After Stroke

Before the injection experiments, VCAM-1 mRNA levels were assessed in vivo at different time points after stroke using quantitative real-time polymerase chain reaction. Our data showed a similar trend to that reported in the literature\(^1\), namely an increase at day 1 with a gradual decline 3 days after stroke (data not shown). Based on our findings, we performed intraarterial injections of the sorted neural stem cells 2 days after stroke. To ascertain the in vivo survival of CD49d positively and negatively sorted stem cells, a control group underwent intraparenchymal stereotactic transplantation. Survival of CD49d-positive and -negative cells after intraparenchymal stereotactic transplantation was not different between the 2 groups (data not shown). Two weeks after intracarotid injection in stroke-damaged animals, NSCs were detected in the perivascular space of capillaries and larger vessels (Figure 4A–B). Some BrdU-positive NSCs in the perivascular space expressed the immature neuronal marker DCX (Figure 4C). In the hemisphere ipsilateral to the stroke, we found a significant number of BrdU-prelabelled NSCs, which had undergone transendothelial migration into the injured brain parenchyma. Cells were found in the cortex (Figure 4F, H) and striatum (Figure 4G) adjacent to the stroke, in the subventricular zone (Figure 4E), and in the hippocampus (Figure 5D). The overall distribution of the cells was not different between the 2 groups. At 2 days after stroke (day of cell injection), we found increased expression of lectin (Figure 4I) and VCAM-1 (Figure 4J–K) in the stroked hemisphere. The distribution of lectin and VCAM-1 expression overlapped with the stroked area (representative MRIs of a typical stroke size and location; Figure 5B) as well as the distribution of the injected stem cells (Figure 5C). Numerous cells in the stroke border zone and the striatum expressed the neuronal marker β-tubulin III (Figure 4G–H). Cells expressing DCX were mainly seen in the cortex (Figure 4F) and the hippocampus (Figure 5D–E). Immature cells expressing nestin were found in the subventricular zone (Figure 4E). To rule out the possibility that the injected NSCs were phagocytosed by monocytic cells in the bloodstream before transmigrating...
into the brain parenchyma, we performed double immunohistochemistry for BrdU and Iba-1 (panmonocytic marker; Figure 4D). We found that only 15% of the BrdU-positive NSCs were also Iba-1-positive in the CD49d-positive group and 10% in the CD49d-negative group. Analysis of cell numbers revealed a significantly more efficient transendothelial migration of CD49d-positive cells as compared to CD49d-negative cells (Figure 5A) in the cortex ($P=0.003$), hippocampus ($P=0.008$), and subventricular zone ($P=0.03$). Representative sections through the hippocampus (Figure 5D–E), the subventricular zone (Figure 5F–G), the cortex (Figure 5H–I), and the striatum (Figure 5J–K) are shown for the CD49d-positive (Figure 5D, F, H, J) and CD49d-negative (Figure 5E, G, I, K) groups. In both the CD49d-positive and -negative groups, NSCs expressed nestin, glial fibrillary acid protein, DCX, and $\beta$-tubulin III (supplemental Figure IA–C, available online at http://stroke.ahajournals.org).

**Behavior**

After stroke, animals showed a drop in their performance on the rotarod as compared with their baseline measurement. Animals injected with vehicle or CD49d-negative cells continued to decline in their rotarod performance, whereas animals injected with CD49d-positive cells started improving at 10 days after stroke. At 17 days after stroke, animals injected with CD49d-enriched cells demonstrated a significantly better sensorimotor recovery compared with animals injected with CD49d-negative cells or vehicle ($P=0.031$; Figure 6A). There was no significant difference between CD49d-negative and vehicle-injected animals. We found a positive correlation between the number of cells and the behavioral performance on a rotarod (Figure 6B, $r=0.51$, $P<0.05$). Analysis of angiogenesis revealed a significantly higher ratio of lectin-positive vessels (comparing stroke penumbra with the contralateral side) in the CD49d-positive group as compared with the CD49d-negative and control groups (Figure 6C–G, $P<0.05$). Analysis of the inflammatory response showed no difference in total number of Iba-1-positive cells. There were significantly more CD68-positive cells and a higher degree of activation (the ratio Iba-1/CD68) in the CD49d-positive group as compared with the other groups (Figure 6H, supplemental Figure II). There was no significant difference between the stroke size and the weight of the animals among the different groups (supplemental Figure III).

**Discussion**

The present study demonstrates that selecting neural stem cells for their expression of CD49d using FACS sorting can significantly increase their homing to the ischemic brain tissue. Improved homing of the CD49d-enriched NSCs to the ischemic brain tissue after intracarotid delivery led to a significantly improved sensorimotor recovery.
Injected neural stem cells reach the target’s vascular bed through chemoattraction followed by rolling, adhesion, diapedesis, and migration20,21 paralleling the mechanism used by inflammatory cells. Cell adhesion molecules, including ICAM-1, ICAM-2, and VCAM-1, are upregulated as part of the inflammatory reaction after stroke with a peak between 24 and 48 hours in rodents in the stroked hemisphere.11 We found high expression of VCAM-1 and lectin at 2 days after stroke (day of cell injection) with a distribution pattern similar to the distribution of stem cells found at 17 days after stroke. VCAM-1 expression at 17 days was very low, which is in accordance with the reported expression profile of VCAM-1 after stroke.11 The ligand to VCAM-1, CD49d, is found on inflammatory cells. We were able to demonstrate dose-dependent adhesion to purified VCAM-1 protein in a static adhesion assay, proving functionality of the cell surface integrin CD49d on the cells we used. In vivo blocking of CD49d with a blocking antibody led to a 39% to 54% reduction in transendothelial migration of neural progenitor cells in an experimental autoimmune encephalomyelitis model,8,10 illustrating the importance of VCAM-1–CD49d interaction for the adhesion and transmigration process. Therefore, increasing the number of cells expressing the integrin CD49d would potentially improve cellular transmigration to the injured brain.

FACS sorting has the advantage of being widely used for clinical purposes, eventually facilitating such an approach for clinical application. In addition to the mechanistic aspect of cell adhesion and migration, the route of delivery plays an important role in the success of cell therapy. Several studies have used the intravenous route to deliver stem cells to the injured brain.5,8–10,22–24 One problem with intravenous injection is the distribution of cells in the whole body, including the liver, spleen, kidney, and the spinal cord. In the study by Pluchino et al, only an estimated 3.1% of the total intravenously injected neural progenitor cells were found in the brain of experimental autoimmune encephalomyelitis mice.10 Liu et al found between 0.75% and 18.5% of the total injected cells (mesenchymal stem cells) in the stroked brain depending on location where counting was done.24 One way to circumvent the whole body circulation is to perform direct intracarotid injection of stem cells.25–27 We found high cell concentrations (up to 1300 cells/mm²) in the areas affected by stroke, including the striatum, cortex, and hippocampus, comparing favorably to cell counts reported in studies with intravenous injection (74 cells/mm² in the study published by Liu et al24).

One limitation of our study is the use of immortalized cells. Although C17.2 cells can illustrate many fundamental aspects of neural biology, their unique properties may limit translation of the reported results to therapeutic application.28 This immortalized cell line has been carefully compared with primary cortical neurosphere-derived neural progenitor cells and the differences have been described in detail.29 An additional caveat is that BrdU was used as a marker for the stem cells. Dilution of BrdU with cell proliferation and leakage of BrdU, especially from dying cells, has been reported.

The application of cell therapy elicits great hope for neurological diseases having only limited therapeutic ap-
proaches. Improved neurological function has been reported in stroke6,24 and experimental autoimmune encephalomyelitis after intravenous infusion of neurosphere-derived multipotent precursors.8,10 Suggested mechanisms include the reduction of inflammatory infiltration, neuromodulation,8,10 or elevation of trophic factors that may be neuroprotective.6 Improved functional outcome after stem cell delivery might be due to induction of angiogenesis in the ischemic tissue.24,25 In our study, we found a positive correlation between the number of cells found in the brain and the behavioral recovery, highlighting the potential importance of more efficient cell delivery. Furthermore, we found a higher degree of angiogenesis in the CD49d group as compared with the other groups. Interestingly, there was also an increased proportion of activated microglia in the CD49d-positive groups as compared with the other groups and its significance warrants further investigation.

We present a concept that potentially improves the efficiency of intravascular stem cell delivery for stroke treatment. We show that selection of a subset of cells that express a specific surface integrin using FACS sorting, a well-established and widely used method, increases the number of homing cells to the lesion and leads to better sensorimotor recovery.

Acknowledgments

We thank Elizabeth Hoyte for preparation of the illustrations.

Sources of Funding

G.K.S. is funded by NIH NINDS 2R01 NS27292, NIH NINDS 2P01 NS37520, the William Randolph Hearst Foundation, Russell and Elizabeth Siegelman, Bernard and Ronni Lacroute, and John and Dodie Rosenkrans. R.G. is supported by the Swiss National Science Foundation, Elizabeth Siegelman, Bernard and Ronni Lacroute, and John and Dodie Rosenkrans. R.G. is supported by the Swiss National Science Foundation PBBEB-104450, SSMSB-1194/PASMA-108940/1.

Disclosures

None.

References


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Stroke. 2008;39:1300-1306; originally published online February 28, 2008;
doi: 10.1161/STROKEAHA.107.500470
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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