Imaging of Stem Cell Recruitment to Ischemic Infarcts in a Murine Model

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Background and Purpose—Neural progenitor cells (NPC) have been reported to aid in the functional recovery from stroke and hold promise as a novel treatment for a variety of neurological diseases. There is a need for imaging tools to study the in vivo migratory behavior of these cells.

Methods—C17.2 NPC, stably transfected with firefly luciferase, were serially imaged through intact skull and skin by bioluminescence imaging over 2 to 3 weeks in nu/nu mice with closed-vessel middle cerebral artery infarcts, followed by contralateral intraparenchymal or intraventricular injections of NPC.

Results—NPC migrated to the site of infarct from the contralateral parenchyma, crossing the midline at 7 days. In control animals without infarcts, NPC remained at the site of administration. Intraventricular cell administration resulted in a wide distribution of cells, including the site of infarct. Within the infarct area, NPC colabeled with the neuronal marker NeuN and with astroglial marker glial fibrillary acidic protein. The time course and magnitude of NPC recruitment were longitudinally compared between the treatment groups.

Conclusions—NPC recruitment to infarct can be assessed noninvasively by serial in vivo imaging. Images correlate well with histological cell distributions. NPC are recruited to infarcts with both parenchymal and cerebrospinal fluid administration, but higher initial photon counts suggest that cerebrospinal fluid administration is more efficient at early infarct seeding. (Stroke. 2004;35:952-957.)

Key Words: diagnostic imaging ■ infarction ■ luciferase ■ stem cells

Stem cell transplantation is a novel potential therapy for ischemic damage and could potentially replace dead neurons and supporting tissue. Several experimental studies have shown improved functional outcome after stroke with stem cell therapy1,2 and fetal graft transplant.3 The mechanisms and means of this functional improvement are currently the focus of much investigation and have created a need for new study methodologies to unravel stem cell–host interactions.

A unique feature of stem cell therapy is the ability of stem cells to migrate to sites of cerebral pathology, first demonstrated in the setting of intracranial gliomas4 but also in the setting of stroke5 and numerous other central nervous system pathologies. This ability likely is centrally important to the reconstitutive effects of stem cell treatments.

Currently, the study of stem cell mobility is limited largely to histological investigation. In vivo stem cell imaging would contribute significantly to the study of stem cells in live animals and ultimately humans. A number of different cell types have been labeled previously with magnetic particles5–8 or gadolinium chelates.9 Cells have also been tracked by isotope and optical methods.10–12 However, there are major unanswered questions regarding stem cell behavior in vivo that cannot be easily addressed with exogenous cell markers. The quantification and proliferation of stem cells cannot be assessed easily in vivo with exogenous markers because they are often degraded, diluted, and excreted as cell populations divide. Endogenous imagable biomarkers such as luciferase,13 by contrast, are expressed in all cell progeny without dilution.

The overall goal of this study was to track the migration of neural progenitor cells (NPC) to sites of ischemic infarct after different routes of injection. These studies have relevance for the eventual potential clinical use of stem cell therapies because the optimal route of administration of such cells and their migratory capability have not been assessed previously. Specifically, we were interested in whether recruitment to infarcts could be demonstrated by bioluminescence imaging. We used NPC C17.2, modified to express the firefly luciferase (Photinus pyralis) marker gene, and a closed-vessel model of middle cerebral artery (MCA) infarction with luciferase bioluminescent imaging to assess the optimal method of stem cell administration.

Materials and Methods

C17.2-LUC-GFP-gal NPC Cell Line

The C17.2 cell line is a clonal, multipotent murine neural precursor cell line, well described previously, that expresses lacZ.14 These
C17.2-gal NPC were stably transfected to express the firefly luciferase (LUC) gene, along with green fluorescent protein (GFP), without changing their stem cell properties.15

Animal Numbers and Study Design
Twenty-five female nude mice (nu/nu; weight, 20 to 25 g) were used for in vivo studies (Charles River Laboratories, Cambridge, Mass). Nine animals underwent focal ischemia with parenchymal injections, 8 animals underwent parenchymal injections of cells without infarcts, and 8 animals underwent intraventricular cell administration with infarcts. All animal studies were conducted according to institutional guidelines.

Stroke Induction and Cell implantation
Focal cortical infarcts16 were surgically induced under ketamine/xylazine anesthesia (90/10 mg/kg IP) by electrocoagulation of the MCA and its main branches. Sham-operated animals were subjected to the same procedure, including craniotomy, but without MCA electrocoagulation. Typical infarct volumes of 18 mm³ were obtained with this method.

Immediately after stroke induction, C17.2-LUC-GFP-gal (1×10⁶ in 4 µL of Hanks’ solution) were stereotactically injected into the left frontal lobe contralateral to the infarct over 30 minutes at the following coordinates: 0 mm anterior, 2.5 mm lateral, 3 mm depth. Control animals (with craniotomies, but without infarcts) received identical cell injections.

Similar to intraparenchymal cell administration, C17.2-LUC-GFP-gal cells (1×10⁶ in 4 µL of Hanks’ solution) were injected into the left lateral ventricle at the following coordinates: 0 mm anterior, 1 mm lateral, 2.5 mm depth.

In Vivo Cell Tracking With Bioluminescence Imaging
Luciferase imaging with the use of C17.2-LUC-GFP-gal cells was performed as previously described.16 Briefly, a custom-built, cryogenically cooled, high-efficiency charge-coupled device (CCD) camera system (Roper Scientific) was used to perform photon counting in anesthetized animals after intraperitoneal injection with β-luciferin (Biosynth; 160 µg/g body wt IP). White light surface images were obtained immediately before each photon-counting experiment to provide an anatomic outline of the animal. Images were acquired from 5 to 20 minutes after β-luciferin administration.

The distribution of luciferase activity within the brain of the animal was then measured by recording photon counts in the CCD. Image processing was performed with a custom-written program. Images were displayed as a false color photon count image superimposed on a grayscale anatomic white light image. Regions of interest were subjected to standard statistical analysis. Imaging was performed at multiple time points (postprocedure days 1, 4, 7, 14, 17, 21); animals were euthanized at days 14 or 21.

Tissue Processing and Immunohistochemistry
Immediately after the final imaging session, mice were killed and brains were harvested. Horizontal 30-µm brain sections (coronal in the mouse frame of reference) were acquired and stored at −80°C until processed for immunohistochemistry.

After the X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; Fisher Scientific) staining, counterstaining with eosin was done or 3,3-diaminobenzidine (DAB) immunohistochemistry was performed. Colocalization of lacZ, luciferase, and enhanced GFP (eGFP) was demonstrated separately in vitro with fluorescent antibody stains.

For immunostaining, the sections were incubated with either NeuN monoclonal antibody (1:20; CHEMICON) or glial fibrillary acidic protein (GFAP; 1:1; DAKO) polyclonal antibody at 4°C overnight in a humid chamber. A biotinylated goat anti-rabbit or horse anti-mouse IgG (1:100; Vector Laboratories) was applied, and the sections were processed with the use of Elite ABC (Vector Laboratories) for 30 minutes, followed by DAB (0.5 mg/mL; Sigma) for 5 minutes.

Results
Serial Histology Showed NPC-LUC-GFP-gal Recruitment to Ischemic Infarct
The C17.2 clone expressed high levels of galactosidase, GFP, and luciferase as determined by fluorescence microscopy and photon counting. Histology obtained 14 and 21 days after contralateral injection showed many NPC clustering in and around the site of infarct (Figure 1a). Some NPC in the infarct demonstrated triangular-shaped neuronal-type phenotypes similar to those observed in pyramidal cells (Figure 1f), with some showing positive staining for the neuronal marker NeuN (Figure 1i). Other infarct-related NPC stained for the glial marker GFAP (Figure 1j). There was a significant population of cells in the white matter tracts between the implantation site and the infarct, suggesting migration from one site to the other. These cells had an elongated shape with leading processes within the white matter tracts of the corpus callosum (Figure 1e), and some stained positive for the glial marker GFAP (Figure 1h). Some NPC remained clustered at the site of the intraparenchymal injection (Figure 1d), where they exhibited a rounded morphology with few processes. A few of these cells stained positive for the neuronal marker NeuN, but the majority of NeuN-positive NPC were found in the infarct itself (Figure 1g).

In parenchymally injected control animals without infarcts, cells generally remained localized to the injection site, but a few cells were observed to move into the ipsilateral subventricular zone, where endogenous neurogenesis is known to occur (Figure 1c).

NPC injected into the ventricle were observed in and around the site of infarct extending from the lining of the cerebrospinal fluid (CSF) spaces to the stroke lesion (Figure 1b). NPC were also noted extensively along the ependymal linings of both ventricles and in the subventricular zone (Figure 1b). Findings suggestive of site-specific differentiation with neuronal-type phenotypes and both neuronal and glial cell markers were observed, similar to the findings in intraparenchymally injected animals.

In Vivo Bioluminescence Imaging Mirrors Histological Findings
There was an excellent correspondence between histological and in vivo bioluminescence imaging with respect to bulk mass of NPC. Figure 2a to 2c shows representative examples of histological sections and corresponding in vivo images (Figure 2d to 2f), done immediately preceding histology.

Intraparenchymal NPC Injections in Infarcted Animals
Both in vivo imaging and histology showed NPC at the site of implantation and crossing the midline through the corpus callosum, with excellent correlation. NPC densely populated the site of infarct in a geographic pattern, sharply delimitated by the extent of the infarct.

Intraparenchymal NPC Injections in Control Animals
There was a single locus of NPC clustering around the site of implantation in a diffuse pattern, with excellent imaging-
histology correlation. Unlike the findings in infarcted animals, there was no sign of contralateral migration and no sign of NPC within the corpus callosum.

**Intraventricular NPC Injections in Infarcted Animals**

There was a wider distribution of cells through the CSF spaces. However, clustering about the site of infarct was again observed. Cells were also noted along the CSF linings and particularly in the subventricular zones.

**Kinetics of Luciferase Signal Migration With Intraparenchymal NPC Injection**

Figure 3a summarizes serial imaging findings in a representative infarcted animal after intraparenchymal NPC injection. Cellular distribution was initially limited to the site of injection, but NPC spread across the midline by day 7 (6 of 8 animals, remainder at 14 days), with population of the infarct by day 7 to 14. The absolute and relative quantitative distribution of luciferase photon emissions (Figure 4a and 4b) showed, after an initial postinjection decrease of signal, a shift of photon emissions from the injected hemisphere to the infarcted hemisphere over time. The right/left ratio first exceeded unity by day 14 (ratio 2.9). This suggests that by the end of the experiment there were more NPC in the infarcted hemisphere than at the injection site because of either massive migration or a combination of migration and in situ proliferation.

**Kinetics of Luciferase Signal Migration in Control Animals Without Ischemic Stroke**

Figure 3b summarizes serial images of a representative control animal. Photon emissions at the site of implantation gradually decreased and did not show migration. This was observed in 5 of 8 animals, with 2 animals transiently showing activity in the right hemisphere and 1 animal showing persistent right hemisphere activity (Figure 4c and 4d).

**Kinetics of Luciferase Signal Migration With Intraventricular NPC Injection Into Infarcted Animals**

Figure 3c shows the imaging findings in a representative animal. Cells initially diffused widely throughout the CSF spaces from the injection site into the right lateral ventricle.
Quantitative analysis (Figure 4e and 4f) again showed an initial postinjection decrease, with a subsequent increase of photon emissions concentrating in the infarcted hemisphere. The right/left ratio exceeded unity by day 7 (ratio 1.2) and continued to rise thereafter, reaching 4.9 by day 14. This indicates higher numbers of NPC in the infarcted hemisphere than in the left hemisphere, where the cells were injected.

Discussion

Our results show that stem cell migration can be studied in vivo and longitudinally in living animals with the use of an endogenous imaging marker gene and bioluminescent imaging. Histological studies confirmed the imaging findings and demonstrated signs of differentiation, as had been shown previously. Imaging demonstrated the following key appearance, consistent with random cell distribution through the CSF. The infarct is populated by day 7 and shows increasing photon emissions thereafter.

Figure 2. Correlation between bioluminescent imaging and histology. X-gal stains with eosin counterstaining (a to c) are compared with false color luciferase photon emission images superimposed on grayscale white light anatomic images (d to f). These were obtained at the end of the longitudinal study (21 days) and are presented in similar orientation (coronal to the mouse). Scale bars = 1 mm. a+d, Intraparenchymal injection of NPC into the hemisphere (white arrow) contralateral to the site of infarction (yellow arrow). Both imaging and histology show the injection site, the cell migration path across the corpus callosum, and the heavily populated infarct. b+e, Intraparenchymal injection of NPC into the hemisphere (white arrow) contralateral to the site of sham surgery (no infarct). Cells are localized to the site of injection, with no distant migration. c+f, Intraventricular injection of NPC (white arrow) contralateral to the site of infarct (yellow arrow). Cells distribute to the subependymal and periventricular areas and to the infarct.

Figure 3. Representative bioluminescent imaging of NPC injections. a, Intraparenchymal injection contralateral to a site of superficial MCA infarction. Cells are initially seen at the injection site and then migrate to the infarct, crossing the midline by day 7 (D7), with subsequent increasing photon emissions at the infarct. b, Intraparenchymal injection into the left lobe of a control animal with sham surgery. Note the decline of photon emissions over time, with eventual disappearance of signal. There is no migration to the contralateral hemisphere. c, Intraventricular injection contralateral to a site of superficial MCA infarction. Note the initial distributed
findings: (1) NPC migrated to an ischemic lesion, but cells implanted in control animals without infarcts showed no migratory behavior. (2) Cells migrated to the infarct, regardless of the method of administration; both intraparenchymal and intraventricular injections yielded equivalent end points. (3) Despite recognizable trends in groups of animals, there was heterogeneity between individuals, easily recognized by imaging in the living animals. (4) There was excellent correlation between imaging findings and histology. A major bottleneck in the in vivo evaluation of stem cell therapies has been the inability to visualize cell populations in the same animal over time. For example, it has been difficult to track the spread of cells or to determine their in vivo expansion or regression. Efficient stem cell therapies in the future are likely to require in vivo monitoring to ensure the following: (1) maximal local cell delivery (percent injected cells) to the area of interest, (2) ubiquitous distribution throughout the area of interest, (3) integration of delivered cells into the host organ structure, and (4) long-term survival (and programmable cell death if necessary) of the delivered cells.

Bioluminescent imaging is useful for answering these questions but is limited by relatively low resolution compared with modalities such as MRI. Bioluminescent imaging should be seen as one of several imaging modalities available to researchers, possibly complementary to other modalities in which higher resolution is required.

We observed changing cellular distributions at serial time points at different locations. Intraparenchymally implanted NPC were composed of 3 groups: (1) cells remaining at the

Figure 4. Quantitative analysis of NPC-related photon emissions. Absolute (left column) and relative (right column) photon counts are shown for the 2 hemispheres. In all cases, note the initial, sharp drop in photon emissions from day 1 through day 4, with subsequent increases in total numbers of photons emitted, particularly in the infarcted animals. Box plots show median as the horizontal mark in the box; upper and lower quartiles define the upper and lower limits of the box; whiskers define largest and smallest values not defined as outliers; outliers are shown as circles and are 1.5 x the interquartile distance beyond the upper or lower quartile bound; extreme values are shown as stars and are 3 x out of bounds. a+b, Intraparenchymal injection with infarct. Note a gradual increase in the right/left ratio of photon emissions over time, indicating the migration of cells from the site of injection on the left to the infarct on the right. The midline is crossed by 7 days. c+d, Intraparenchymal injection with sham surgery. Cells remain confined to the left hemisphere at the site of the injection, with little or no migration. e+f, Intraventricular injection with infarct. Note the bilateral distribution of cells on initial imaging, indicating wide distribution through the CSF, with subsequent increases in photon emissions over the site of infarct.
implantation site, (2) cells populating the ischemic and penumbral regions, and (3) cells located in white matter between these regions. Immediately after NPC implantation, virtually all cells were at the injection site. At 7 days a considerable number of cells were seen crossing the midline through the corpus callosum. Analysis of bioluminescence data suggests that approximately 40% of total cells at 7 days were contralateral to the site of injection after intraparenchymal administration. This would correspond to a speed of migration of the leading edge of approximately 360 μm/d (15 μm/h). A previously reported migratory rate of 65 μm/h has been observed in an infarct model, whereas 120 μm/h was reported for the normal trafficking of progenitor cells from the subventricular zone to the olfactory bulbs of rodents. At 14 days, more NPC cells had reached the infarct site, with a further increase by 21 days. At the latter time point, approximately 70% of all cells were localized in the ischemic hemisphere as determined by imaging and confirmed by X-gal histological staining.

NPC population kinetics were different for intraventricular administration. Cells dispersed within CSF on injection showing a wider initial distribution. While bioluminescence imaging could not be used to clearly distinguish between photons originating from ipsilateral ventricles versus ischemic regions, X-gal staining showed populations of NPC in the peri-infarct regions at 14 and 21 days. Effective trans-CSF seeding has also been observed in other studies.

The specific chemotactants responsible for NPC homing are not completely known. Preliminary evidence suggests that some of the major stimuli are SDF-1 and reelin.

C17.2 NPC have previously been shown to participate in neural development and to be capable of differentiation into diverse neuronal and glial cell types as well as normal cytoarchitectural constituents. Our own observations confirm this, with clear neural and astrocytic differentiation (Figure 1). These results again demonstrate the extraordinary capability of NPC to migrate to infarcts and participate in repair. Both intraparenchymal and intraventricular routes of NPC administration gave similar favorable results, but the data suggest that intraventricular injections result in earlier infarct population. It is unclear at this point whether this earlier infarct population results in clinical benefit.

In the future, NPC may be further engineered to express trophic factors, may target specific tissues, or may be controlled with tetracycline-dependent promoters. Many of these studies would likely benefit from imaging feedback with bioluminescent imaging.

Acknowledgments

Dr Kim was supported in part by the Brain Korea 21 project. Dr Schellingerhout was supported in part by grants from NIH 2P01CA6924605, 1R24CA97282-01, the Radiologic Society of North America, and The American Brain Tumor Association. The authors gratefully acknowledge the work of Drs Yi Tang and Yoshi Saeki in the preparation of the C17.2-LUC-GFP-gal cell line. The original C17.2 cell line was a kind gift from Dr Evan Y. Snyder. Dr Edward Graves was responsible for the development of our custom software package, CMIR-Image, without which our data analysis would have been nearly impossible.

References

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*Stroke*. 2004;35:952-957; originally published online February 26, 2004;
doi: 10.1161/01.STR.0000120308.21946.5D
*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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