The Postischemic Environment Differentially Impacts Teratoma or Tumor Formation After Transplantation of Human Embryonic Stem Cell-Derived Neural Progenitors

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Background and Purpose—Risk of tumorigenesis is a major obstacle to human embryonic and induced pluripotent stem cell therapy. Likely linked to the stage of differentiation of the cells at the time of implantation, formation of teratoma/tumors can also be influenced by factors released by the host tissue. We have analyzed the relative effects of the stage of differentiation and the postischemic environment on the formation of adverse structures by transplanted human embryonic stem cell-derived neural progenitors.

Methods—Four differentiation stages were identified on the basis of quantitative polymerase chain reaction expression of pluripotency, proliferation, and differentiation markers. Neural progenitors were transplanted at these 4 stages into rats with no, small, or large middle cerebral artery occlusion lesions. The fate of each transplant was compared with their pretransplantation status 1 to 4 months posttransplantation.

Results—The influence of the postischemic environment was limited to graft survival and occurrence of nonneuroectodermal structures after transplantation of very immature neural progenitors. Both effects were lost with differentiation. We identified a particular stage of differentiation characterized in vitro by a rebound of proliferative activity that produced highly proliferative grafts susceptible to threaten surrounding host tissues.

Conclusion—The effects of the ischemic environment on the formation of teratoma by transplanted human embryonic stem cell-derived neural progenitors are limited to early differentiation stages that will likely not be used for stem cell therapy. In contrast, hyperproliferation observed at later stages of differentiation corresponds to an intrinsic activity that should be monitored to avoid tumorigenesis. (Stroke. 2010;41:153-159.)

Key Words: brain transplantation ■ human embryonic stem cells ■ neural differentiation ■ stem cell therapy ■ stroke ■ teratoma

The potential of stem cell transplantation for the treatment of acute brain lesions has been highlighted by a number of experimental results. Among available cell sources, embryonic stem cells (ESCs) offer advantages related to their main attributes, pluripotency, and self-renewal. However, both properties come with major drawbacks that will also apply to induced pluripotent stem cells that express ESC-like characteristics.1

Pluripotency can generate teratoma, a tissue characterized by the presence of mature derivatives of at least 2 of the 3 germ layers.2 Occurrence of teratoma was reported independently of the origin of the ESC, that is, mouse,3,4 nonhuman primate5 or human,6,7 and with allografts8 or xenografts,9,10 although a higher incidence was reported in allograft situations.8 Although the question has been controversial,11 ESCs may generate nonmalignant teratoma rather than malignant teratocarcinoma12 that is characterized by the presence of cells that indefinitely maintain pluripotency. To avoid teratoma, much effort has been devoted to the elaboration of differentiation protocols that allow maximal homogeneity of the transplant6 or to cell sorting before transplantation to eliminate nonneural progenitors.13-15

A more recent series of studies have reported a different risk of tumor formation after transplantation of ESC derivatives into the brain,6,15,16 which corresponds to maintained proliferation of differentiated progenitors. This process, also referred to as “graft overgrowth,” is characterized by the exclusive presence of neural cells, therefore excluding the
classification as teratoma, that may provoke compression and disruption of host brain structures.

A major issue raised by those results is the relative role of intrinsic and environmental factors in the development of adverse structures in the host brain. It is unclear whether this development is only linked to the persistence of poorly differentiated cells into the graft or whether, in addition, dedifferentiating/proliferative signals may be released by the lesioned brain. Acute stroke involves strong inflammatory and glial reactions with elevated contents of key cytokines in human ESC (hESC) amplification and differentiation such as basic fibroblast growth factor\(^1\) and brain-derived neurotrophic factor.\(^2\) The possibility that these factors induce a reversal of the commitment of ESC derivatives and/or favor cell proliferation is, therefore, of particular importance for cell therapy to patients with stroke.

We have analyzed the influence of the ischemic environment on the formation of teratoma/tumors by transplanted hESC-derived neural progenitors (NPGs). Human ESCs differentiated toward the neural lineage with limited instructive cues were transplanted into brains with no, small, or large middle cerebral artery occlusion lesions. We show that the postischemic environment influences the formation of teratoma formed by NPG in their early stages of differentiation. The possibility that these factors induce a reversal of the commitment of ESC derivatives and/or favor cell proliferation is, therefore, of particular importance for cell therapy to patients with stroke.

Transcriptional Analysis

Total RNA was isolated with the RNeasy Mini Kit (Qiagen). One microgram mRNA was reverse-transcribed with SuperScript II (Invitrogen). Real-time quantitative polymerase chain reaction was performed in triplicates with the Power SYBR Green PCR Mix (Applied Biosystems) and a Chromo 4TM Real-Time amplifier (Bio-Rad). The amplification efficiency of each primer pair (Table) was determined with a standard curve from fetal brain cDNA (Clontech). Quantification was performed at a threshold detection line (Ct value). The Ct of each target gene was normalized against that of the 18S housekeeping gene. Gene relative expression levels were determined with the 2\(^{-ΔΔCt}\) method with hESC cDNA as a calibrator. Data are expressed as mean±SEM.

Ischemia and Transplantation

All animal experimentation was performed on compliance of the European ethical guidelines (86/609/EEC). A total of 192 adult (250 g) male Sprague-Dawley rats (Charles River, L'Arbresle cedex, France) were used in 2 separate experiments (n=102 and n=90, respectively). In each experiment, animals were randomly allocated to 3 groups to receive a transient middle cerebral artery occlusion\(^1\) with isoflurane anesthesia (Figure 1). In one third of the animals, the filament was immediately removed (sham group). The striatal lesion group (STR) received a 90-minute occlusion, and the STR cortical lesion group (STR-Cx) a 2-hour occlusion. Animals were followed for body weight and potential postoperative complications during 48 hours and were transplanted 7 days postsurgery.

NPGs were transplanted weekly along their differentiation process from 21- to 49-div (n=6 for each time point and each lesion group) and then at 86-div (n=6 for each lesion group). In the first experiment, 2 additional animals in each lesion group were transplanted with either hyperproliferative NPG (or late-NPG; Figure 2; n=6 in total) into the lateral ventricle or with detachable embryoid body-like structures present in early NPG cultures (Figure 3A; n=6 in total). Animals received a daily administration of cyclosorpin A (8 mg/kg, intraperitoneally, Sandimmun; Novartis), azathioprine (2 mg/kg, subcutaneous, Immurel; GlaxoSmithKline), and methylprednisolone (2 mg/kg, intraperitoneal, Solu-Medrol; Pfizer) starting the day before transplantation and for the whole duration of the experiment. Four animals died after transient middle cerebral artery occlusion surgery (one in the sham and 3 in the STR-Cx group) and 2 additional animals died after transplantation (one in the sham and one in the STR group).

![Figure 1](http://stroke.ahajournals.org/)

Figure 1. Representative examples of the 3 types of environment. A, Sham: no lesion. B, STR: loss of the lateral striatal quadrant. C, STR-Cx: loss of the whole middle cerebral artery territory. Volume reconstruction from 12 cresyl violet sections at 180-μm interval. Percentages indicate the volume of the ipsilateral hemisphere (Hemisph.) or striatum to the contralateral (contralat.) tissue.
Magnetic Resonance Imaging
MRI was performed using a 4.7-T Bruker spectrometer equipped with a custom made surface coil. A T2-weighted turbospin echo sequence RARE (repetition time TR = 3000 ms, effective echo time TE = 36.0 ms, turbo factor = 8, number of acquisitions AC = 8, field of view = 3.5 cm, matrix 256 × 256, slice thickness 0.85 mm, acquisition time 9 minutes 36 seconds) was used, providing quality transversal/coronal anatomic images.

Tissue Processing
Rats were terminally anesthetized after 1 or 4 months with 100 mg/kg sodium pentobarbital (Ceva) and were perfused with phosphate-buffered 4% paraformaldehyde. Three rats in the STR group transplanted with 86-div cells were perfused after 3 months. Cryostat-cut 30-μm-thick brain sections were stained with Cresyl violet for reconstruction of lesion volumes (Free-D software, Amib, France; Figure 1) or processed for immunohistochemistry. Antibodies were rabbit polyclonal to glial fibrillary acidic protein, nestin (Chemicon), doublecortin (Abcam), human DARPP-32 (Santa-Cruz Biotechnology), Pax6, and β-tubulin III (Covance), mouse IgG to human nuclei (Chemicon), Oct4 (Santa Cruz Biotechnology), cytokeratin-14 (Chemicon), rat tyrosine hydroxylase, MAP2 (Sigma); and mouse IgM to Ki67 (Sigma). Secondary antibodies were Alexa488- and Alexa555-labeled (Molecular Probes; 1:1000) and 4’,6-diamidino-2-phenylindole was used to visualize nuclei. Controls were performed with the corresponding isotypes. Observations were performed on a Zeiss Axioplan microscope with ImageJ (http://rsb.info.nih.gov/ij) for image analysis.

Results
Pretransplantation Differentiation Profile
The differentiation profile of SA-001 hESC was divided into 4 stages on the basis of the expression of pluripotency, proliferation, and maturation markers (Figure 2). As compared with levels recorded in undifferentiated hESC, early NPG (21- to 28-div) still contained high levels of Oct4 (34.6% ± 6.6%), Nanog (37.8% ± 6.1%), and Sox2 (23.3% ± 4.5%) and of the proliferation markers Ki67 (93.8% ± 9.2%) and proliferating cell nuclear antigen (198.6 ± 2.5%). Mid-NPG (35-div) no longer expressed ESC markers (Oct4: 0.6% ± 0.3%; Nanog:...
Neural markers Pax6 (42- to 49-div) were characterized by strong expression of the neuronal marker levels close to fetal brain.

Considering the lack of consensus in the literature about the terminology of ESC-derived structures after transplantation, we have followed Nature Biotechnology recommendations.

Tumor Formation
A main characteristic of late NPG-derived grafts was the multiplication of rosettes containing numerous Ki67-labeled cells (Figure 4J–K). Rosette-rich grafts developed independently of the lesion presence or size, including after transplantation into the lateral ventricle (Figure 4M). After 1 month, they ranged in size from 1 (n = 7) to 3 mm (n = 1, lateral ventricle group). Although no compressions of surrounding structures were observed in late NPG-grafted animals, examples of compressive tumors have been described after longer posttransplantation delays.7,14 Tumors were composed only of neural cells.

Differentiation and Integration Into the Host Parenchyma
In agreement with their pretransplantation commitment, mid-NPG grafts contained doublecortin-migrating neuroblasts (Figure 4H) and MAP2 neurons sending long processes through the corpus callosum (Figure 4I). Host-derived glial fibrillary acidic protein astrocytes were seen overlying the grafts (Figure 4G). Interestingly, host astrocytes would not invade nonneural and nondifferentiated structures, indicating recognition of the graft content. Glial fibrillary acidic protein-positive astrocytes of graft origin were present only in the 4-month-old grafts derived from NP (Figure 4N–Q) in parallel with DARPP-32 (Figure 4O–P), a phosphoprotein enriched in the dopaminergic striatal neurons. No teratocarcinomas were observed after transplantation of NPG at any differentiation stage. In contrast, invasive teratomas with mature tissues from the 3 germ layers consistently formed after transplantation of the embryoid body-like structures present in early NPG cultures (Figure 3; 6 of 6 animals). They could be identified with T2 MRI by their nonhomogenous aspect. They ranged from 3 to 6 mm in diameter and occurred independently of the lesion (Figure 3C–F). Embryoid body-like structures are eliminated during the second passage (30-div) and were no longer present in mid-NPG.

Discussion
The main result of this study is the identification of 2 different origins for adverse events after transplantation of hESC-derived NPG. The first mechanism, teratoma formation, depends...
on environmental cues because it increased after grafting into the postischemic parenchyma. The second mechanism, graft overgrowth, is independent on the environment and relates to the existence of a peak of intrinsic proliferative activity that occurs at a later stage of differentiation.

Ischemia Influences Teratoma/Hyperproliferation Formation by Early NPG
The observed impact of the postischemic environment on teratoma formation may relate to the strong expression of antiapoptotic factors after stroke, which can promote both graft survival and teratoma formation, or of factors known for their role in self-renewal, like basic fibroblast growth factor. As indicated by the quantitative polymerase chain reaction analysis, early NPG grafts still contained high levels of markers of pluripotency and proliferation. Rather than a dedifferentiating effect of the lesion, this suggests a contamination of the grafts by cells with limited commitment that responded to environmental cues present in the postischemic brain as they did in vitro before transplantation. An increased occurrence of teratomas has been reported in allograft versus xenograft of undifferentiated or lightly differentiated cells. Although poorly differentiated progenitors must definitely be excluded from stem cell therapy products, preclinical studies

Figure 4. Fate of transplanted NPG and NP. A–E, Early NPG develop into nonneuroectodermal structures (A, cartilage), and into mature ectodermal (B, cystic epithelium; inset shows the alignment of single human (human nuclei, green) cells; C, cytokeratin-14 keratinocytes) and neural (D–E) cell types. Arrow in D indicates the border of a cystic cavity. F–I, Mid-NPG contain rosettes (nestin staining in F) that generate large amounts of neurons (G) migrating into the host parenchyma (doublecortin staining in H) and sending processes through corpus callosum (cc in I) and into the cortex (c). J–M, Late NPG grafts contain numerous rosettes (J, inset shows a mitosis) with proliferative centers (K). Host astrocytes avoid rosettes (L, compare with G). Rosette-rich grafts in the lateral ventricle (M). N–Q, Four months after transplantation, graft-derived astrocytes (N) and DARPP-32 neurons (O–P) are observed in NP-derived grafts. DARPP-32 neurons form patches that receive host tyrosine hydroxylase afferents (Q, section adjacent to P).
with allografts of extensively differentiated progenies are still needed.

The ischemic parenchyma also releases a number of cytokines and factors that have protective effects on neurons, including vascular endothelial growth factor and endothelial nitric oxide synthase produced by endothelial cells or glia-derived neurotrophic factor produced by astrocytes. Neural progenitors express receptors for basic fibroblast growth factor. The prosurvival effect of brain lesions has been reported. However, it was no longer significant after differentiation into NPs, which apparently differ from early NPG in their sensitivity to extrinsic trophic factors or to transplantation.

In contrast with a previous report, transplantation into the adult ischemic brain did not essentially modify the fate of hESC-derived progenitors. Nonneural cell types and Oct4-positive cells were found after transplantation of early NPG containing high levels of Oct4 mRNA. Groups of rosettes were observed after transplantation, including into the lateral ventricles, of late NPG that contained highly proliferative rosettes. The delayed appariation of NP-derived glial fibrillary acidic protein-positive cells in the graft after transplantation of NP was consistent with a normal neurogenesis process. Discrepancies between the 2 studies might come from the status of the host (neonate versus adult) or from accelerated in vitro differentiation in a culture medium greatly enriched compared with ours.

Overgrowth

Besides the possibility of teratoma formation, hESC-derived grafts pose a risk for extensive graft growth that can lead to compression and necrosis of the surrounding tissue. Overgrowths have been reported in grafts from human fetal tissue and from hESC-derived rosettes. In our study, large grafts were exclusively neural composed of rosettes that generated impressive amounts of nestin-positive progenitors and TuJ1-positive neurons at the expenses of the host tissue. Overgrown grafts occurred exclusively after transplantation of NPG that were characterized in vitro by a high proliferative activity after the second passage. This suggests that late NPGs constitute a particular maturation stage comparable to one of orchestrated waves of proliferation observed during normal central nervous system development.

Conclusions

This study shows that hESC-derived NP develop according to, and at the pace of, their own developmental program after transplantation into the rodent ischemic brain. The impact of ischemia on the formation of nonneural tissues is limited to poorly differentiated populations that are not suitable for stem cell therapy. In contrast, the proliferation profile must be monitored to avoid overgrowth after transplantation. These 2 newly revealed phenomena should be taken into account in setting up programs of cell therapy for stroke and, more generally, for brain disorders.

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Disclosures

None.

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