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SCIENCE DIRECT
Experimental Neurology 199 (2006) 339–347
Experimental Neurology
www.elsevier.com/locate/yexnr

Sustained survival of xenografted human neural stem/progenitor cells in experimental brain trauma despite discontinuation of immunosuppression

André Wennersten [a,*], Staffan Holmin [a], Faiez Al Nimer [a], Xia Meijer [a], Lars U. Wahlberg [b], Tiit Mathiesen [a]

[a] *Department of Clinical Neuroscience, Section of Clinical CNS Research, Karolinska Institutet, S-171 76 Stockholm, Sweden*
[b] *NS Gene A/S, Copenhagen, Denmark*

Received 26 June 2005; revised 22 December 2005; accepted 29 December 2005
Available online 21 February 2006

Abstract

Neural stem cells have emerged as a promising therapeutic tool in CNS disease and injuries. In the clinical setting, cultured human neural stem/progenitor cells (hNSC) are an attractive possibility for transplantation to the damaged brain. However, transplantation of hNSC requires toxic immunosuppressive treatment to avoid rejection. The aim of the current study was to evaluate if shortening the duration of immunosuppression by cyclosporin A would affect hNSC survival and differentiation after transplantation to the site of a focal brain injury in the rat. hNSC were xenografted to the *hippocampus* and the medial limit of an experimentally induced cortical contusion. The animals received immunosuppression for either 6 or 3 weeks or no immunosuppression. The status of the grafted human cells was analysed by immunohistochemistry. No statistically significant differences were observed between the two immunosuppressed groups regarding graft survival, migration or proliferation at 6 weeks post-transplantation. In contrast, the graft survival was extremely poor in the non-immunosuppressed group. Furthermore, the expression of the differentiation markers nestin, neuronal nuclei (NeuN) and glial fibrillary acidic protein (GFAP) in the transplanted cells did not differ significantly between the two immunosuppressed groups. Moreover, a fourth group of eight animals that were immunosuppressed for 3 weeks were allowed to survive for 6 months. Five of these rats demonstrated robust graft survival in the *hippocampus* and scattered cells in the cortex. This study demonstrates the importance of immunosuppression but also the possibility of shortening immunosuppression without impacting on the phenotype of the grafted hNSC.

Keywords: Fetal stem cell; Traumatic brain injury; Immunosuppressed; Neural progenitor; Immunesuppressed, xenotransplantation; Xenogeneic; Cyclosporine

Introduction

Transplantation of neural stem cells has shown considerable therapeutic potential in several animal models of central nervous system (CNS) disorders (Aboody et al., 2000; Deacon et al., 1998; Ehtesham et al., 2002; Ogawa et al., 2002; Philips et al., 2001; Riess et al., 2002; Vescovi et al., 1999a,b). Recent findings points towards cultured human derived stem cells as an attractive choice for clinical applications due to the possibility of obtaining significant number of cells from a limited amount of material (Carpenter et al., 1999; Flax et al., 1998). Although there is an extensive body of data building up on the use of transplanted human neural stem/progenitor cells (hNSC) in several models of CNS injury, including stroke and traumatic brain injury, studies on the effect of different amounts and duration of immunosuppression are still scarce (Jeong et al., 2003; Kelly et al., 2004; Svendsen et al., 1996; Wennersten et al., 2004). Immunosuppressive agents have potentially toxic side effects for the patient. Today, most experimental xenografting protocols use high doses of immunosuppressant throughout the lifespan of the animals (Deacon et al., 1998; Ishibashi et al., 2004; Kelly et al., 2004; Le Belle et al., 2004; Wennberg et al., 2001). A more precise knowledge on the effect of immunosuppressive treatment on the grafts and the mechanisms preceding rejection in the CNS may prevent unnecessary suppression of a patient's immune system. Furthermore, the potential benefit of a shortened period of

* Corresponding author. Fax: +46 8 517 717 78.
E-mail address: andre.wennersten@ki.se (A. Wennersten).

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doi:10.1016/j.expneurol.2005.12.035

immunosuppression is that long term experimental studies will be greatly facilitated. Data from behavioral testing would be easier to interpret due to the substantial reduction of stress imposed on the animals due to the immunosuppression. Finally, the susceptibility to infections would be decreased.

We have previously demonstrated that cultured and cryopreserved hNSC transplanted to an experimental contusion injury in the rat survive, proliferate and differentiate 6 weeks post-grafting. In that study the animals received cyclosporin A during the entire experiment (Wennersten et al., 2004).

The current study was undertaken to determine if the duration of immunosuppression by cyclosporin A could be decreased without reducing survival or affecting the phenotype of hNSC grafted to the traumatically injured brain.

Material and methods

Generation and in vitro culture of human neural stem/progenitor cells

7-week-old post-conception forebrain tissue was recovered from an elective first trimester routine abortion using the regular vacuum aspiration technique. The collection of residual tissue was approved by the Human Ethics Committee of the Huddinge University Hospital, Karolinska Institute, and was in accordance with the guidelines of the Swedish Society of Medicine including an informed consent from the pregnant women seeking abortions. The preparation of the tissues was done in accordance with previously published protocols (Carpenter et al., 1999). Briefly, the collected tissue was micro dissected in sterile saline and transferred to N2 medium, a defined Dulbecco's modified Eagle's medium (DMEM)/F-12 medium including 0.6% glucose, 2 mM glutamine, 5 mM HEPES buffer, insulin (25 μg/ml), transferring (100 μg/ml, Sigma), progesterone (20 nM, Sigma), Putrescine (60 μM, Sigma), Selenium chloride (30 nM, Gibco) and 2 μg/ml heparin. The tissue was triturated in DMEM/F12 medium using a glass/Teflon Potter-Elvehjelm homogenizer. The viable cells were counted with tryphan blue exclusion and the volume was adjusted by the addition of culture medium to obtain a cell density of 250,000 cells/ml. The cells were grown in the N2 medium supplemented with recombinant human epidermal growth factor (EGF; 20 ng/ml, R&D Systems), recombinant human basic-fibroblast growth factor (bFGF; 20 ng/ml, R&D Systems), and recombinant ciliary neurotrophic factor (CNTF; 10 μg/ml, R&D Systems). The cells grew as free-floating clusters (neurospheres), were passaged by mechanical dissociation every 10 days and reseeded as single cells at a density of 100,000 cells/ml. The cells for transplantation were frozen at passage 9 and recultured until passage 10 before the transplantation.

Preparation of cells for transplantation

The human neural stem/progenitor cells were taken for transplantation 4–5 days after the last passage as small spheres. The spheres were collected by centrifugation at 1000 rpm for 3 min and resuspended in 1 ml DMEM/F12 medium. The sphere suspension was centrifuged again and resuspended in a smaller volume to give the equivalent of 100,000 cells/μl.

Surgical procedures

36 male Sprague–Dawley rats weighing approximately 300 g were anesthetized by intramuscular injection of Hypnorm (fluanisonum, 10 mg/ml and fentanylium, 0.2 mg/ml, Janssen, Belgium) and Dormicum (midazolam, 1 mg/ml, Roche). In addition, 0.1 ml of Xylocain-Adrenalin (lidocaine hydrochloride, 5 mg/ml and adrenaline, 5 μg/ml, Astra, Sweden) was injected subcutaneously in the sagittal midline of the skull before the skin incision. The rats were put into a stereotactic frame and a craniotomy was drilled 3.0 mm posterior and 2.0 mm lateral bregma. A standardized parietal contusion was made by letting a 24-g weight fall onto a rod with a flat end diameter of 1.8 mm from a height of 6.0 cm. The rod was allowed to compress the tissue a maximum of 3.0 mm. 30 of the rats received hNSC while 6 received hNSC that had been killed by repeated freezing and thawing. Ten minutes after the injury, the cultured hNSC or killed hNSC were injected to the medial margin of the contusion (3.0 mm posterior and 1.1 mm lateral to the bregma). 1 μl was injected at a depth of 3.5 mm and 1.1 μl was injected at a depth of 2.0 mm from the cortical surface. The animals were treated with subcutaneous injections of cyclosporin A (Sandimmun, Novartis, Sweden) 4 mg/kg every Monday and Wednesday and 8 mg/kg every Friday for immunosuppression starting the day before surgery and with trimethoprim 12 mg and sulphadoxin 60 mg (Borgal Vet, Intervet, Stockholm, Sweden) to 500 ml of drinking water for infection prophylaxis. 6 animals did not receive any immunosuppression (6 weeks survival), 19 animals received cyclosporin A for 3 weeks (6 weeks or 6 months survival) and 11 animals received cyclosporin A for 6 weeks (6 weeks survival). The 6 animals that were transplanted with dead hNSC were divided between the 3 and 6 week immunosuppression group (6 weeks survival). The 6-month animals were kept in the animal care facility under standard conditions after the cessation of immunosuppression. All the animals were sacrificed by decapitation under identical anaesthesia as during the initial surgery 6 weeks or 6 months after the transplantation. The brains were immediately dissected and frozen in isopentane containing dry ice, 14 μm coronal cryosections were cut serially and stored at −20°C.

Immunohistochemistry

After thawing at room temperature, the sections were fixed in formaldehyde. Endogenous peroxidase was quenched for 30 min in 0.5% hydrogen peroxide diluted in PBS. The sections were incubated at room temperature for 1 h with PBS containing 1% bovine serum albumin, 0.3% Triton X-100 and 0.1% sodium azide and avidin block solution (Vector Laboratories Inc., Burlingame, CA, USA) to prevent non-specific binding. This solution, without the avidin, was used for all subsequent