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Transplantation of TAT-Bcl-x_L-transduced neural precursor cells: Long-term neuroprotection after stroke

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ABSTRACT

Neural precursor cells (NPC) are an interesting tool in experimental stroke research, but their therapeutic potential is limited due to poor long-term survival. We therefore *in vitro* transduced subventricular zone-(SVZ)-derived NPC with the anti-apoptotic fusion protein TAT-Bcl-x_L and analyzed NPC survival, differentiation, and post-stroke functional deficits after experimental ischemia in mice. Survival of TAT-Bcl-x_L-transduced NPC, which were injected at day 7 post-stroke into the ischemic striatum, was significantly increased at 4 weeks after stroke. Increased survival of NPC was associated with reduced infarct injury and decreased post-stroke functional deficits. Animals grafted with TAT-Bcl-x_L-transduced NPC showed an increased number of immature cells expressing the neuronal marker doublecortin. Since mature neuronal differentiation of NPC was not observed, reduced post-stroke injury cannot be attributed to enhanced neuronal regeneration, but rather to indirect by-stander effects of grafted NPC. In line with this, NPC-mediated neuroprotection of cortical neurons *in vitro* was associated with increased secretion of growth factors. Thus, *in vitro* transduction of cultivated NPC with TAT-Bcl-x_L results in enhanced resistance of transplanted NPC followed by long-term neuroprotection and ameliorated functional deficits after transient focal cerebral ischemia in mice.

Introduction

Endogenous neurogenesis persists in the adult rodent brain within the subventricular zone (SVZ) of the lateral ventricle (Gross, 2000; Taupin and Gage, 2002). The germinal niche consists of at least three cell types, which are astrocyte-like neural precursor cells (NPC; type B cells) that differentiate into rapidly dividing transit amplifying cells (type C). These cells in turn give rise to neuroblasts (type A; Doetsch et al., 1999b). Thereafter, neuroblasts migrate via the rostral migratory stream to the olfactory bulb where they differentiate into interneurons (Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1999a).

Endogenous neurogenesis of the adult brain is not only observed under physiological conditions but is also affected by various pathological stimuli including cerebral ischemia. Increased post-ischemic proliferation of SVZ-derived cells as assessed by 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU) incorporation followed by migration towards the lesion site and subsequent neuronal differentiation have been shown in various models of cerebral ischemia (Liu et al., 1998; Zhang et al., 2001; Arvidsson et al., 2002; Li et al., 2002; Yamashita et al., 2006). However, the extent of neuronal differentiation or integration of new-born $BrdU^+$ cells seems to be very low taken into account that the majority of these cells die within weeks after the ischemic onset (Parent, 2003; Haas et al., 2005).

Since *endogenous* neurogenesis plays a minor role in post-stroke recovery due to the impaired survival of new-born cells, new strategies and "therapeutic" approaches in experimental cell-based stroke research have evolved. Among these, *exogenous* adult NPC have been applied to further support post-ischemic *endogenous* neurogenesis. The advantage of this approach is an easier accessibility of adult NPC with minor ethical questions compared to the application of embryonic stem cells. In culture, SVZ-derived NPC show a high proliferative activity forming so called neurospheres (Bez et al., 2003) that give rise to neurally differentiated cells such as mature astroglial or neuronal cells both *in vitro* and *in vivo* (Cattaneo and McKay, 1990; Alvarez-Buylla and Lois, 1995).

Although several studies showed an amelioration of post-stroke injury and reduction of functional deficits after NPC transplantation, these effects were often limited to subacute time points and neuronal replacement of lost neurons by transplanted cells has not been convincingly shown, yet (Toda et al., 2001; Zhang et al., 2003; Hicks et al.,

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2008). About 99% of transplanted NPC are supposed to die within weeks after transplantation (Hicks et al., 2008). We therefore followed a different "therapeutic" approach by combining a cell based therapy with a neuroprotective treatment in order to improve the poor survival of transplanted NPC. In the present study, SVZ-derived NPC were *in vitro* transduced with the anti-apoptotic and membrane-permeable fusion protein TAT-Bcl-x_L (Dietz et al., 2002) before stereotactically transplanted into the ischemic striatum of mice. Thereafter, the extent of ischemic injury, functional outcome as well as cell proliferation and differentiation of transplanted and endogenous NPC were studied for up to 4 weeks after transplantation.

Materials and methods

Animals and experimental groups

Experimental procedures were performed according to the European Communities Council Directive (86/609/EEC) for the care and use of laboratory animals and approved by local authorities. For induction of cerebral ischemia adult male C57Bl/6N mice (Charles River, Germany; 11–13 weeks old) weighing 22–27 g were used and assigned to 9 groups as depicted in Table 1. Treatment assignment was performed in a blinded manner. Each group consisted of 8–10 animals. All animals were intraperitoneally injected with BrdU (Sigma, Germany; daily dose: 50 mg/kg bodyweight) starting on day 8 after stroke until the end of the experiment. For *in vitro* assays preparation of NPC was performed on 6- to 8-week-old male C57Bl/6 N mice (Charles River, Germany) whereas for *in vivo* application of NPC transgenic green fluorescence protein positive (GFP^+) animals (C57BL/6-Tg ACTB-EGFP, 1Osb/J; JAX Laboratory, Bar Harbor, Maine; male, 6–8 weeks old) were used. GFP expression in these animals was dependent on the actin promoter, which allows reliable tracking of transplanted NPC. Generation of cortical neurons was performed on E18 embryos from pregnant Wistar rats.

Preparation and culture of SVZ-derived neural precursor cells

Preparation and subsequent culture of NPC was done according to a modified protocol from Rietze and Reynolds (Rietze and Reynolds, 2006). Briefly, NPC were isolated from the SVZ of 6- to 8-week-old mice. The SVZ was micro-dissected under stereomicroscopic control (Zeiss, Germany) and minced into small pieces, and then mechanically triturated and dissociated into a single cell suspension. Cells were cultured in serum-free basic medium DMEM-F12 (PAA, Austria) supplemented with epidermal growth factor (EGF, 2 μg/ml), basic fibroblast growth factor (bFGF, 2 μg/ml), and penicillin-streptomycin (Invitrogen, Germany). Cells were incubated with 5% CO_2 at 37 °C. The growth factors were added every 2–3 days and the cells were passaged via accutase (Invitrogen, Germany) digestion for 30 min at 37 °C with re-suspension every 10 min. Thereafter, cells were centrifuged and re-suspended in pre-warmed conditioned media. The neurosphere passage was done every 7–10 days and the cells used for transplantation were harvested after passages 2–3. For TAT-Bcl-x_L transduction, NPC were incubated under standard cell culture conditions for 4 h with TAT-Bcl-xL (1 μM). Before stereotactic injection, the cells (both transduced and native) were washed with PBS to remove remaining extracellular TAT-Bcl-x_L in cell culture medium.

Table 1
Experimental treatment design.

	PBS	NPC (native)	NPC (transduced)
Survival time	7 days	7 days	7 days
	14 days	14 days	14 days
	28 days	28 days	28 days

Mice were assigned to 9 treatment groups (8–10 animals per group) and underwent induction of 30-min middle cerebral artery occlusion (MCAO). Seven days after MCAO PBS, native neural precursor cells (NPC) or TAT-Bcl-x_L-transduced NPC were stereotactically injected into the ischemic striatum. Survival times given refer to day of stereotactic surgery, which was performed on day 7 after induction of cerebral ischemia. For proliferation analysis, all animals were i.p. injected with BrdU starting on day 8 after transplantation until the end of the experiment.

Preparation of cortical neurons

For preparation of cortical neurons, pregnant Wistar rats were sacrificed by CO_2 intoxication at embryonic day 18. Embryos were dissected, tissue pieces trypsinated, and dissociated using a fire-polished Pasteur pipette. Cells were seeded on poly-L-ornithine/laminin (Sigma, USA) coated glass cover slips at a density of 100,000/cm^2. Cultures were maintained at 37 °C in a humidified atmosphere at 5% CO_2 in a culture medium that was based on Neuroblast medium (Gibco, USA) containing additional Transferrin (Sigma, Germany), PSN (Gibco, USA), L-Glutamine (Seromed, Germany) and B27 supplement (Gibco, USA). For OGD assays, cultured cells were used on day 3 after preparation and the medium was changed 24 h before induction of OGD.

Expression and purification of Bcl-x_L and TAT fusion proteins

The pTAT-HA and pTAT-Bcl-x_L vectors including a histidine tag and a hemagglutinin (HA) sequence were previously described (Nagahara et al., 1998; Dietz et al., 2002). The protein purification procedure has also been described in detail before (Dietz and Bähr, 2007). Briefly, TAT-HA and TAT-Bcl-x_L were expressed in *Escherichia coli* strain BL21 (DE3) pLysS (Novagen, USA) and lysed by sonication. Lysates were denatured in 8 mol/L urea before affinity chromatography. Bacterial debris was pelleted and the supernatant was subjected to metal-affinity chromatography using a Ni-NTA matrix (Qiagen, Germany). Salt and urea were removed by gel filtration on Sephadex G-25 M (Amersham Pharmacia Biotech, Piscataway, NJ), using a buffer containing 10 mmol/L Tris, 50% glycerol, 0.1 mmol/L EDTA, 0.1% pluronic acid and 0.02% Tween 80. After dialysis against two changes of 10 mmol/L Tris, pH 10.0, 274 mmol/L NaCl, the protein solution was removed from the dialysis tubing and centrifuged for 5 min at 15,000*g*. The supernatant was removed and protein concentration determined by comparison with a protein standard by SDS-PAGE after Coomassie staining.

Detection of TAT-Bcl-x_L in transduced neural precursor cells in vitro

Detection of TAT-Bcl-x_L in transduced cultured NPC was done by Western blotting using an antibody against the HA sequence of the recombinant protein. Cells were pre-incubated for 4 h with or without TAT-Bcl-x_L (1 μM). After repeated centrifugation, removal of the supernatant and washing, the cells were lysed with lysis buffer containing 50 mM Tris at pH 8.0, 150 mM NaCl and Triton 1%. The lysates were centrifuged and supernatants used for SDS-PAGE. For Western Blot analysis equal amounts of protein (40 μg) were diluted in 6× sample buffer, boiled and loaded onto 12% polyacrylamide gels. TAT-Bcl-x_L (1 μM) was loaded as positive control. Proteins were transferred onto PVDF membranes, which then were immersed in blocking solution (5% milk in TBS-T (0.1% Tween 20 + TBS); 1 h at RT) and incubated with a monoclonal mouse anti-HA antibody (Covance, USA; 1:1000 in TBS-T with 1% milk; 18 h at 4 °C). Subsequently, membranes were incubated with a peroxidase-coupled, goat anti-mouse secondary antibody (1:2000; Santa Cruz, Germany), washed several times, immersed in ECL solution and exposed to ECL-Hyperfilm (Amersham, Germany).

Oxygen-glucose-deprivation (OGD) test

NPC were passaged and 100,000 cells were used for each condition. In order to pre-incubate cells with TAT-Bcl-x_L, they were first incubated in conditioned medium with 1 μM TAT-Bcl-x_L for 4 h and