The effect of grafted neuroectodermal stem cells on injured spinal motoneurons following ventral root avulsion and reimplantation: insights into the molecular mechanism

Ph.D. thesis

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Introduction

Injury to the spinal cord often results in irreversible loss of function. Adult motoneurons survive axonal injuries inflicted far away from the cell body. However, most of the adult motoneurones die when their axons are damaged close to the some. A ventral root avulsion from the spinal cord induces degeneration of motor axons, loss of synapses and death of motoneurons. This process is due to a cascade of events involving activation of astrocytes and microglial cells and the excessive amounts of excitotoxic glutamate release in the injured cord.

Neurotrophic factors, pro- and anti-inflammatory proteins are critical mediators of the post-traumatic reactions. Neurotrophic factors regulate motoneuron survival and appear to be able to induce endogenous regenerative processes. Cytokines released by the central nervous system and microglia play key roles in the regulation of inflammatory reactions and in activation of the inflammatory cells.

Various therapeutic approaches aiming to treat and alleviate the consequences of spinal cord injuries involve neurotrophins, particularly GDNF and BDNF produced by grafted stem cells. Although neurotrophins induce motoneuron survival they do not promote axonal regeneration.

In our study we used a clonal neuroectodermal stem cells line (NE-GFP-4C) isolated from brain vesicles of embryos of transgenic mice lacking the tumor suppressor gene p53. The aim of the present study was to analyse and compare the therapeutic potential of transplanted NE-GFP-4C murine neuroectodermal cells applied in topically different transplantation paradigms and determine the molecular mechanisms of the neuroprotective action of grafted neuroectodermal stem cells responsible for the motoneuron-rescuing effect.

Material and methods

The lumbar 4 (L4) ventral root of Sprague-Dawley rats was avulsed and reimplanted ventrolaterally into the injured cord. Neuroectodermal stem cells were injected immediately following avulsion injury into the L4 segment (ARG group), into the reimplanted ventral root (ARG-IR group) or were placed in fibrin clot around the reimplanted root (ARG-PR group). The control experiments involved rats whose L4 root was avulsed and reimplanted without a stem cell graft (AR group). Three months after the primary surgery rats were set up for tension recording or CatWalk gait analysis. Motor units of Extensor Digitorum Longus (EDL) and Tibialis Anterior (TA) were determined in the ARG and AR groups. The L4 motoneuron pool was retrogradely labelled with Fast Blue and the numbers of reinnervating motoneurons were determined in all groups 3 months after the surgery. Expression of various immune factors (IL-1-alpha, IL-1-beta, IL-6, IL-10, MCP-1,

MIP-1-alpha, MCSF, and TNF-alpha) and neurotrophic factors (BDNF, GDNF, NT-3, NT-4, PDGF-alpha, PDGF-beta, PTN, and TGF-beta) expected to prevent motoneuron death in the grafted cord was determined by PCR and immunohistochemistry in short term experiments.

In experiments using neutralising antibodies a mini-osmotic pump filled with a mixture of function-blocking antibodies against MIP-1a, IL-1a, IL-6, TNF-alpha and IL-10 was placed subcutaneously in the dorsal region. In another set of experiments osmotic pumps filled with IL-10 antibody only were used. Control animals received pumps filled with identical volumes of antibody-specific IgG isotypes only. The animals survived for 3 months and were then processed for motoneuron counts and histological analyses.

Results

Animals that received intraspinal stem cell grafts (ARG group) have 70% of their L4 motoneurons regenerated into the vacated endoneural sheaths of the reimplanted root. Morphological reinnervation was accompanied by significant functional recovery (number of retrogradely labelled cells: 711 ± 14 [ARG]. Intraradicular neural stem cell grafting (ARG-IR group) resulted in good morphological and functional reinnervation (number of retrogradely labelled cells: 671 ± 26 [ARG-IR]), while both negative controls and animals with perineural stem cell treatment showed poor motor recovery (number of retrogradely labelled cells : 65 ± 2.5 [ARG-PR] and 42 ± 10 [AR]).

Stem cell grafts produced the modulatory cytokines IL-1-alpha, IL-6, IL-10, TNF-alpha and MIP-1-alpha, but no neurotrophic factors. The neurons and astrocytes in the ventral horn of grafted animals also produced IL-6 and MIP-1-alpha. The infusion of function-blocking antibodies against all cytokines into the grafted cords completely abolished the motoneuron-rescuing effect, while neutralization of only IL-10 suggested its strong effectivity as concerns motoneuron survival and a milder effect on reinnervation.

To determine the factors acting in the grafted cords, we performed a series of semiquantitative PCR analyses of the segment L4 in ARG and AR animals. It emerged that on postoperative days 2, 5, 10 and 14 there was no difference between the AR and ARG animals (n=4 in each group) in the mRNA levels of the investigated neurotrophic factors (BDNF and GDNF), but the mRNA levels of the interleukins IL-1-alpha, IL-6 and IL-10, TNF-alpha and MIP-1-alpha were significantly higher in the ARG than in the AR animals, typically at 5 and 10 days following grafting. However, the mRNA production of these factors had declined by 14 days after grafting.

To distinguish between graft and host immune factor production, we used the laser microdissection technique to perform a qPCR analysis on identical parts of spinal cord sections (ventral horns) taken from AR and ARG animals and from the stem cell graft. The stem cell grafts and the ventral horns

of the ARG animals produced considerable amounts of the cytokines investigated, with appreciable increases by 10 days as compared with 5 days after grafting; IL-10 proved to be an exception, as it was produced in greater amounts by the grafted cells 5 days after grafting. In contrast, the ventral horns of the AR animals produced increased amounts of the mRNAs of these factors at 5 days, with a moderate decline by 10 days after the avulsion injury; IL-10 was again an exception: it was not produced in the control cords at all. The cultured NE-GFP-4C cells (native graft source) did not display detectable levels of the mRNAs of any of the factors. At a survival time of 10 days, the ventral horns of the ARG animals exhibited increased mRNA levels of all the factors, including IL-10, relative to the mRNA levels of the AR animals, suggesting a graft-induced upregulation of these factors at the mRNA level in the host cord.

Through the use of mouse-specific antibodies, strong immunoreactivity to all five factors was found to be exerted by the grafted cells at 5 days after grafting. It was noteworthy that the majority, but not all of the grafted cells were immunopositive for the cytokines tested. However, at 10 days postoperatively only the strong expression patterns of IL-6, TNF-alpha and MIP-1-alpha were maintained; the immunofluorescence of IL-1-alpha and IL-10 was confined to some of the stem cells located at the periphery of the graft. On the other hand, immunohistochemistry with anti-rat/mouse specific antibodies indicated that the host ventral horn neurons and glial cells of the ARG animals appeared to produce only IL-6, IL-10 and MIP-1-alpha. The expression patterns were uniform at both 5 and 10 days after grafting. Confocal microscopic analysis of double-labelled sections revealed that the glial IL-6, IL-10 and MIP-1-alpha reactivity was confined to the astrocytes, and not to the microglia/macrophages of the host cord, even though the degree of astrogliosis in the ARG animals was limited. A similar distribution pattern of these cytokines was observed in the AR animals, but the astroglial density was increased. Despite the relatively high mRNA levels of IL-1-alpha and TNF-alpha, no immunoreactivity to these factors could be detected as compared with the biological positive controls.

To test whether these cytokines are indeed responsible for inducing the prevention of motoneuron death, for 2 weeks we used osmotic pumps to infuse the mixture of function-blocking mouse-specific antibodies produced against all five factors to the grafts. The infusion of function-blocking antibodies against all cytokines into the grafted cords completely abolished their motoneuron-rescuing effect (57 ± 5 reinnervating cells), while neutralization of only IL-10 suggested its strong effectivity as concerns motoneuron survival and a milder effect on reinnervation (195 ± 10 reinnervating motoneurons). In order to test whether the application of an osmotic pump induces a damage in the rescued motoneuron pool, pumps filled with isotype-specific IgGs were used and no significant difference relative to the ARG animals was found (695±44 reinnervating cells).

Discussion

In this study we have provided evidence, that grafted embryonic neuroectodermal stem cells are able to rescue the vast majority of damaged motoneurons otherwise destined to die. However, rescue of injured motoneurons by grafted stem cells was only successful if the stem cells were placed into the affected segment of the spinal cord or into the reimplanted ventral root of animals whose L4 ventral root was avulsed to bring about motoneuron death.

Furthermore, cytokines produced by grafted neuroectodermal stem cells are likely to rescue damaged motoneurons following ventral root avulsion injury via two distinct mechanisms. First, the diffusible factors that modulate the environment of injured motoneurons are able to reach the ventral horn from more remote positions provided they are located still within the boundaries of the spinal cord and related spinal roots. At least some of the cytokines, especially IL-10 are thought to exert a direct neuroprotective effect on the injured neurons themselves.

Second, the intraspinal neuroectodermal stem cell transplantation significantly decreased astroglia and microglia reactions within the affected L4 segment of ARG animals rostrally and caudally from the injury site at 5 and 10 days after grafting compared with controls.

These data suggest that the anti- and pro-inflammatory cytokines have a strong modulatory function in the CNS, that promotes the prevention of neuronal cell death and induces regeneration.

Original papers related to this thesis

I. Pajenda G, **Pajer K**, Márton G, Hegyi P, Redl H, Nógrádi A. Rescue of injured motoneurones by grafted neuroectodermal stem cells: Effect of the location of graft.
Restor Neurol Neurosci. 2013 Jan 1;31(3):263-74. doi: 10.3233/RNN-120294.
Impact factor: 4,179

II. **Pajer K,** Feichtinger GA, Márton G, Sabitzer S, Klein D, Redl H, Nógrádi A. Cytokine signaling by grafted neuroectodermal stem cells rescues motoneurons destined to die.
Exp Neurol. 2014 Jun 5;261C:180-189. Impact factor: 4,617 (2013)