Environment-dependent fate of implanted neural stem cells in the brain

PhD theses

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Introduction

’In adult [brain] centers, the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated.’ Ramon y Cajal wrote in 1913. His ‘harsh decree’ was gradually effaced by the facts retrieved from the scientific experiments of the 1960-90’-s. The progress in neuroscience showed that **neurogenesis** is maintained in the adult CNS, but is restricted to some defined regions. Despite of the sustained neurogenic capability, the **regeneration capacity of the adult brain tissue is not sufficient to repair major damages**. New neurons evolve in low numbers, and new neuronal networks are rarely organized in the adult brain. The treatment of CNS disorders and lesions represent a particular challenge.

In theory, CNS regeneration can be promoted by the recruitment of stem cells from endogenous pools or by implanting exogenous stem cells, those capable of generating neurons in the lesion area.

Stem cells are able to renew themselves and in the meantime, to produce progenies differentiating to mature tissue-type cells. Certain tissue stem cell populations can preserve proliferation capacity throughout the life-span of the organism. In the adult body, a number of different stem cell populations exist. Toti-, multi-, or bipotent stem cells can be distinguished according to the number of cell types they can generate.

Adult neurogenesis occurs in the **subventricular zone** (SVZ) [lining the lateral ventricles at the striatum] and the **subgranular zone** (SGZ) of the hippocampal dentate gyrus. Neuroblasts migrating along the rostral migratory stream (RMS) from the subventricular zone to the olfactory bulb (OB), participate in the replacement of olfactory neurons, and contribute to the adaptation to new olfactory stimuli and the development of olfactory memories. The neuroblasts of the subgranular zone differentiate into granule cells and integrate into the granular layer of hippocampus. New granule cells may have roles in learning and memory processes. While the observations derive mostly from rodent models, neurogenesis is known also in the adult human brain.

Subventricular and subgranular zone, RMS and OB are ‘**neurogenic regions**’. They support the proliferation (SVZ, SGZ), migration (RMS) or differentiation (OB) of stem cells. These regions were shown to help the survival and region-specific differentiation of exogenous stem

cell derivatives, as well. In other, ‘non-neurogenic regions’, however, exogenous stem cells can not survive for long time and their differentiation results only in glial cell types, without any significant neuron formation.

Besides adult neurogenesis in the SVZ and SGZ in ‘intact’ mammals, cell production can be provoked by different pathological conditions in the subependymal layer throughout the CNS. While cell-proliferation and migration of neural precursors were observed in pathological conditions, region-specific differentiation and integration were rarely seen in the adult brain. Cellular and axonal regeneration is inhibited by microenvironmental factors characteristic to the mature brain parenchyma, which are substantially different from those in neurogenic zones. The creation of a ‘permissive’ brain environment is indispensable for the survival and integration of either endogenous or exogenous stem cells, and the tissue-type development of their derivatives.

The level of oxygen supply is an important environmental factor which regulates the outcome of neural tissue damages. By changing the local oxygen tension, the fate of implanted stem cells can be altered, as well. The links between hypoxia and stem cell fate were already analysed in myogenic and neural differentiation processes. The effects of the actual oxygen-level on developing cells at defined phases of differentiation, however, have not been evaluated.

The use of **hyperbaric oxygen** (HBO) therapy has been suggested decades ago as a neuroprotective adjuvant therapy. During the treatment, patients inhale 100% oxygen at elevated (2-3 ATA) pressure. As a result, the oxygen concentration rises in the body fluids and can provide tissue oxygenation in the areas of definitive damage. CNS disorders are often accompanied with certain degree of tissue hypoxia. The correction of hypoxia may help the regeneration of primary lesions and prevent the evolution of secondary damages (e.g. those in stroke penumbra regions). We hypothesized that oxygenation may support the differentiation of neurons from endogenous or exogenous stem cells, as well.

Cell therapy – in theory - promise new neurons, astrocytes and oligodendrocytes for damaged brain areas. Besides replacement of decaying cells, implanted cells may serve as vehicles for gene delivery, may secrete neurotrophic factors and induce angiogenesis, can influence immune reactions and inflammatory processes. Also, they can create a more permissive microenvironment for regeneration and endogenous neuron production. For any

potential beneficial actions, however, stem cells require permissive environment for survival, controlled proliferation and tissue integration.

Cells from many resources can be used for implantation. Embryonic stem cells, immortalized, one-cell derived stem cell lines, freshly isolated stem/progenitor cells, fetal tissue blocks, adult stem cells have been used in implantation experiments. Valuable studies need cells, which are available in high numbers at the same quality, can give rise to tissue-friendly progenies, can be identified after implantation, and may have potential to integrate or at least to survive in the damaged brain areas.

For successful cell therapies, an understanding on the permissive brain environment and its interactions with appropriate stem/progenitor cells, have to be achieved.

Objectives

In my thesis work, multiple effects of the host brain tissue on the fate of implanted neural stem cells had been investigated. I implanted geno- and phenotipically identical, cloned neuroectodermal stem cells into the forebrain cortices of adult mice at various pathophysiological conditions.

I intended to answer the following questions:

How can we modify the microenvironments of the adult brain in order to enhance its receptivity for multipotent neural stem cells and their derivatives? How can we modify the commitment of stem cells to make them capable of integration to the brain?

I. Does the environment of lesioned brain areas influence the fate of implanted stem cells differently in comparison to the intact brain tissue?
II. What sort of interactions may form between stem cells and tumour cells either in vitro or in vivo?
III. Can the intracerebral integration of stem cells be facilitated by implanting cells after in vitro induction of neural tissue-type commitment?
IV. How does environmental oxygen tension influence the survival, proliferation and neural differentiation of stem cells?

Methods

A. In vitro methods

1. Maintenance of neural stem cells
2. Recultivation of implanted GFP-4C cells from host brains
3. Establishment of primary astroglial cell cultures
4. Maintenance of glioma and glioblastoma cell lines
5. In vitro induction of neural development of stem cells by retinoic acid treatment or by co-culturing with astrocytes
6. Hypoxic treatment of cells
7. Examination of cell adhesive properties by chimera aggregation technique
8. Assays on cell proliferation by [^{3}H]-thymidine incorporation technique
9. Determination of the chromosome number
10. Viability assays by MTT and AlamarBlue techniques
11. Examination of gene expression by RT-PCR method

B. In vivo models

1. Establishment of pathologic animal models: cryogenic cortical lesion, brain tumour
2. Cell implantation into different regions of the adult mouse brain
3. Hyperbaric oxygen treatment
4. Behavioural tests: “sticky tape”, “tight rope”, “forelimb asymmetry” tests
5. Transcardiac perfusion

C. Sample processing and data elaboration

1. Fixation, freeze-sectioning
2. Immunocytochemical, histological stainings
3. TUNEL-reaction
4. Fluorescent and confocal microscopy
5. Statistical probes

Results

In the presented experiments, cells of the NE-4C neuroectodermal stem cell clone and its subclones (GFP-4C, PLAP-4C) carrying identifiable marker genes were used. We studied the survival, proliferation and differentiation of stem cells in intact and modified brain environments, as well as under different in vitro conditions.

- Fate of implanted neural stem cells in intact and freeze-lesioned mouse brain cortices

The data demonstrated that the environment of the intact adult forebrain does not support long-time survival, neuronal differentiation and integration of implanted neural stem cells. The lifespan of implanted stem cells in the intact forebrains was restricted to less than 6 weeks, except in the subventricular zone, where a few non-differentiated cells survived longer. In contrast, lesioned cortical environment supported the long-time (>8 weeks) survival of implanted stem cells and facilitated their migration into the cell-deficient lesion zone. While the astrocyte-type differentiation of grafted cells was not inhibited, neuronal differentiation did not take place in the lesioned cortex. Significant alterations of intrinsic cell properties, those potentially elicited by the different tissue environment were excluded by cell biological analyses on cells recultivated from the host brains. The data proved, that the environment instructs an altered fate of stem cells in lesioned and intact brain cortices.

- Stem cell - tumour cell interactions

In vitro data showed that NE-4C stem cells establish direct cell-to-cell contacts with certain (GL261 glioblastoma), but not with (U87 glioma, C6 glioma) all neural tumour cell types. Moreover, the proliferation of certain tumour cell types (C6 glioma, LL glial cells) is promoted by soluble factors produced by stem cells. The media of the investigated tumour cells, on the other hand, had no effect on the proliferation of NE-4C stem cells. The data indicate that application of stem cells as vehicles for anti-cancer drugs may hold the risk of tumour progression in some combinations of tumour-stem cell pairs.

The presence of GL261 glioblastoma cells ‘permitted’ the survival of NE-4C stem cells inside the brain, but the growth of tumour cells significantly exceeded the growth of stem cells. Stem cells could not migrate toward the tumour from a distance of millimetres indicating that tumour cells did not secrete (enough) diffusible factors to facilitate stem cell migration toward the tumour mass (‘tumour-targeting’).

- In vitro commitment to neural differentiation did not promote the integration of stem cells either into intact or lesioned cortical areas

Committed neural progenitor state of differentiation was induced in vitro by exposing GFP-4C stem cells to retinoic acid or by co-culturing with astrocytes. In vitro, these treatments result in the formation of neurons and astrocytes in characteristic times after the induction. In the environment of brain cortices however, pre-differentiated GFP-4C cells could generate only a few astrocytes: the early commitment-stage of implanted stem cells was not enough to initiate neuron formation and tissue integration inside the brain. In contrast, pre-differentiated progenitors produced tumour-like structures, sporadically, in the host brains. According to data on cells recultivated from the expanding cell mass, the tumours could be originated either from host or from implanted cells, but with altered intrinsic cellular properties. These facts call the attention to the probability of tumour formation, one of the most important dangers of cell therapy.

- The effects of different oxygen levels on the fate of NE-4C neural stem cells

Effects of in vitro hypoxia on NE-4C cells

A 48-hour exposure to hypoxia did not modify the viability and growth of non-committed neural stem cells. The same hypoxia or that applied in later stages (days 2-4, 4-6, 6-8) of neural differentiation, however, resulted in significant changes in the fate of differentiating progenies.

The number of neurons by the 8^{th} day of induction dropped most drastically if cultures were exposed to hypoxia on days 0-2 or 6-8. The data indicated that the sensitivity to hypoxia highly depends on the stage of neural differentiation. The most sensitive periods include the stage of neuronal commitment of stem cells (days 0-2) and the stage of neuronal maturation of postmitotic neuronal precursors (days 6-8). Behind the phenomenon, a significantly reduced expression of proneural and neural genes was detected in response to hypoxia.

Hyperbaric oxygen treatment enhance the probability of intracerebral neuron formation by grafted stem cells

Brain disorders and lesions are often accompanied (or caused) by tissue hypoxia. Cryogenic brain lesion leads to hypoperfusion and consequential tissue hypoxia. On the bases of in vivo results, we hypothesized that hypoxia contributes to the failure of neuron production by

implanted neural stem cells. For a partial correction of tissue hypoxia we applied hyperbaric oxygen treatment (HBOT) on animals previously exposed to cortical lesion and stem cell implantation.

HBOT evoked important changes in the fate of both implanted and host cells. The elevated oxygen level reduced the accumulation of host cells in the lesion zone and reduced the volume of implanted cell-mass in the lesion area, as well. TUNEL assays and the evaluation of mitotic frequency revealed, that HBOT decreases the rate of both apoptosis and proliferation. In spite of the reduction in cell number, in HBO-treated cortices, some of the implanted cells could develop into neurons. The data indicated that hyperbaric oxygen treatment made the environment more permissive for neuronal-type cell differentiation.

Conclusions

My intention was to reveal some important parameters of the host brain environment, which may profoundly influence the fate of implanted neural stem cells, those capable to generate mature neurons. For this end, I modified the host environment and implanted stem cells at different stages of in vitro induced neural development.

I proved that cortical lesions – in contrast to the intact parenchyma - provide adequate environmental signals for growth and migration of non-differentiated neural progenitors of both exogenous and endogenous origin, but do not support the formation of neurons. Applying hyperbaric oxygenation, we demonstrated that hypoxia is one of the non-permissive factors which hinder the neuronal differentiation of implanted stem cells.

Integration of neuronal progenitors and maturing neurons into the brain tissue requires complex cooperation between the host environment and the grafted cells. The circumstances ideal for the maintenance and proliferation of stem cells are profoundly different from the conditions necessary for the integration of postmitotic neurons.

It is of utmost importance for the effective and secure future cell therapies, to understand precisely the interactions of cells in different commitment phases with the local microenvironment, and to gain information on the requirements of cells at different level of neural development for survival, differentiation and integration.

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Publications

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