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PERSPECTIVE

Immune remodeling of stromal cell grafts in the central nervous system: therapeutic inflammation or (harmless) side effect?

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Abstract

Over the past two decades, several cell types with fibroblast-like morphology, including mesenchymal stem/stromal cells, but also other adult, embryonic and extra-embryonic fibroblast-like cells, have been brought forward in the search for cellular therapies to treat severe brain injuries and/or diseases. Although current views in regenerative medicine are highly focused on the immune modulating and regenerative properties of stromal cell transplantation *in vivo*, many open questions remain regarding their true mode of action. In this perspective, we integrate insights gathered over the past 10 years to formulate a unifying model of the cellular events that accompany fibroblast-like cell grafting in the rodent brain. Cellular interactions are discussed step-by-step, starting from the day of implantation up to 10 days after transplantation. During the short period that precedes stable settlement of autologous/syngeneic stromal cell grafts, there is a complex interplay between hypoxia-mediated cell death of grafted cells, neutrophil invasion, microglia and macrophage recruitment, astrocyte activation and neo-angiogenesis within the stromal cell graft site. Consequently, we speculate that regenerative processes following cell therapeutic intervention in the CNS are not only modulated by soluble factors secreted by grafted stromal cells (bystander hypothesis), but also by *in vivo* inflammatory processes following stromal cell grafting.

Key words:

stromal cells, transplantation, neuroinflammation, neuroprotection, graft-remodeling

Introduction

During the past two decades, pre-clinical research aiming to cure severe neurological injuries has witnessed a spectacular increase of highly promising experimental therapeutic interventions, jointly referred to as “stem cell therapy” (Stoltz, et al., 2015). Various multipotent cell types, including adult mesenchymal stem/stromal cells (MSC) and adult/embryonic/extra-embryonic fibroblast-like cells, are investigated in experimental cellular therapies to overcome the nearly irreversible nature of brain injuries (Dulamea, 2015, Rivera and Aigner, 2012). As *ex vivo* culture-expanded MSC and other fibroblast-like cells (independent of their origin) are phenotypically and functionally indistinguishable they will be referred to as stromal cells further on in this perspective (Costa, et al., 2015, Hematti, 2012, Jones, et al., 2007, Mueller and Coles, 2014). While numerous studies provide evidence for the beneficial effects on neuropathology by stromal cell grafting in the CNS (Jaramillo-Merchan, et al., 2013, Lee, et al., 2010, Yoo, et al., 2013, Zanier, et al., 2014), to date the true mode of action remains unknown. To shed light on the underlying mechanisms, it is not only necessary to investigate the effects exerted by grafted cells on CNS function/physiology, but it is equally important to focus on the response of the host niche to the cell graft (Costa, et al., 2015, De Vocht, et al., 2013, De Vocht, et al., 2013, Le Blon, et al., 2014, Praet, et al., 2014). In this perspective, we try to create a new model focusing on the bidirectional interplay of cellular events following stromal cell grafting in the CNS of mice, integrating current literature with our own efforts over the past 10 years.

Immune remodeling of stromal cell grafts in the central nervous system.

Although neglected for many years, it is now well established that direct grafting of both autologous (syngeneic) and allogeneic/xenogeneic stromal cells, including MSC and embryonic/extra-embryonic fibroblast-like cells, in brain tissue induces a severe immunological response. While xenogeneic and allogeneic stromal cell grafts become rapidly rejected upon grafting in immune competent hosts (Camp, et al., 2009, Ronsyn, et al., 2007, Tambuyzer, et al., 2009), grafting of syngeneic stromal cells seems to be well tolerated (Coyne, et al., 2007, De Vocht, et al., 2013, Praet, et al., 2012) despite complex immune remodeling within and surrounding the graft site (Le Blon, et al., 2014, Praet, et al., 2014). Here, we will provide a step-by-step overview of cellular events that occur following syngeneic stromal cell grafting into brain tissue starting from the day of implantation until 10 days after implantation. We consider this 10-day period to be the most critical time frame for stromal cell grafts to settle in - for them - the non-natural brain environment.

Entry routes to the CNS: stromal cell administration

Several routes of administration can be applied for stromal cell delivery to the (injured) CNS. Although for practical reasons intravenous (iv) injection would be preferred in current clinical settings, this route strongly relies on the original assumption that stromal cells can migrate to the site of injury in the CNS via intrinsic expression of multiple homing receptors (Cornelissen, et al., 2015, Eggenhofer, et al., 2014). However, various studies have demonstrated that iv administered stromal cells are unable to reach the CNS in sufficient numbers to be of clinical relevance, due to cell retention in lung

capillaries, the spleen or lymph nodes (Acosta, et al., 2015, Jackson, et al., 2010, Reekmans, et al., 2011). Although iv administered stromal cells may exert immunomodulating effects on peripheral immune cells in those retention tissues, only to migrate to the lesion sites in the CNS at a later time point and induce an indirect beneficial effect on neuropathology (Morando, et al., 2012, Salinas Tejedor, et al., 2015), current clinical trials for multiple sclerosis and amyotrophic lateral sclerosis have not yet provided a proof-of-principle for successful iv administered stromal cell therapy in human pathology. So far, only safety and tolerability of stromal cell injection have been validated by these clinical trials (Lublin, et al., 2014, Martinez, et al., 2012, Oh, et al., 2015, Prockop, et al., 2014). However, several studies comparing various administration routes of stromal cells have observed a superior effect when cells were injected at the targeted location (Moscoso, et al., 2009, Paul, et al., 2009, Seo, et al., 2011). Therefore, potential alternative routes for cell delivery to the injured CNS are intrathecal, intraventricular, intracerebral (comprising multiple regions) or intraspinal injection. Based on our own experience, we will here further discuss the cellular remodeling events following intracerebral implantation of stromal cells (see Figure 1) (Bergwerf, et al., 2009, De Vocht, et al., 2013, Le Blon, et al., 2014, Reekmans, et al., 2012).

The day of intracerebral stromal cell grafting: hypoxic stress

Conceptually, intracerebral injection of a stromal cell suspension consists of a precise slowly timed mechanical injection of minute volumes of cells into the CNS, thereby subtly pushing and possibly damaging the surrounding tissue. Immediately following injection, the stromal cell graft will present itself as a bolus of viable cells entrapped within the host's tissue (Figure 1A) (Praet, et al., 2014). Due to the absence of blood

vessels within the cell graft, the core of the stromal cell graft will be subjected to severe hypoxic stress within the first hours post-grafting (Figure 1B) (De Vocht, et al., 2013, Praet, et al., 2014). Although detrimental for the stromal cell graft itself, this natural feature is not necessarily negative in terms of therapeutic potential, as stromal cells under hypoxic conditions are known to alter their gene expression and the secretion of paracrine factors (Page, et al., 2014, Zhu, et al., 2006). For instance, expression of vascular endothelial growth factor (VEGF) is upregulated, which is involved in the induction of angiogenesis, while secretion of monocyte chemotactic protein-1 (MCP-1), which is involved in the chemotaxis of monocytes towards the site of injury/disease, and matrix metalloproteinase-2 (MMP-2), which is involved in the breakdown of extracellular matrix, is decreased (Page, et al., 2014). These hypoxia-induced alterations in gene/protein expression by cellular grafts might possibly act as pro-survival and/or neuro-protective signals for the injured brain (Plotnikov, et al., 2013, Tong, et al., 2015), although this hypothesis will need further confirmation *in vivo*.

Day 1: Early infiltration of neutrophils

Despite grafted stromal cells reside under hypoxic conditions for the first 24 hours, which may generate a beneficial effect on neuro-repair, it is also well-known that severe oxidative stress on cells will lead to caspase-dependent apoptosis (Zhu, et al., 2006). As a consequence of this hypoxic and most likely also nutrient-deprived environment, 24 hours after cell implantation the core of a stromal cell graft will be highly apoptotic and necrotic, leading to a very early influx of neutrophils (Figure 1C). The influx of neutrophils is by no way surprising as the direct injection of a stromal cell graft in the CNS will inevitably cause a disruption of the blood-brain-barrier in proximity of the

graft-site and needle tract, a feature also observed following sham-injection (Praet, et al., 2014). Whether this disruption is only temporary still needs to be defined. Furthermore, as at this stage a large portion of grafted cells will be apoptotic and necrotic, cell debris in the core of the implant will give rise to a large amount of damage-associated molecular patterns (DAMPs), e.g. heat shock proteins (HSP), ATP, nucleic acids, consequently being an additional driving force for attracting neutrophils (Caielli, et al., 2012, Vernon and Tang, 2013).

Day 3: Second phase of immune cell invasion and first sign of neo-angiogenesis

By day three post-implantation, neutrophils will have cleared most of the cell debris present within the core of the stromal cell graft. This is also the stage where several major changes will occur within the stromal cell graft in the CNS (Figure 1D). First, the size of the cellular graft will strongly decrease as all necrotic tissue will be cleared, with less than an average of 20% of the initial grafted cell number remaining (De Vocht, et al., 2013). Second, once neutrophils have exerted their phagocytic function, they will start expressing several soluble mediators. These include so-called ‘find-me’-signals, which serve as a tracking signal for phagocytic leukocytes, such as microglia and macrophages (Martin, et al., 2015, Vernon and Tang, 2013). Next, neutrophils may be killed via death receptor-induced apoptosis, as both infiltrating macrophages and brain-resident microglia are able to release death receptor ligands, e.g. TNF α and Fas-ligand (Geering and Simon, 2011, Martin, et al., 2015). In line with this, at day three post-implantation phagocytic leukocytes, microglia and/or macrophages, are abundantly present at the graft site, most likely in order to phagocytose apoptotic neutrophils and/or remaining cellular debris (Denes, et al., 2007, Neumann, et al., 2008). Note that it is

extremely difficult to distinguish between both phagocytic cell populations, especially in wild type mice (Durafourt, et al., 2012, Hickman, et al., 2013). Third, while stromal cells can produce VEGF under hypoxic conditions (Page, et al., 2014), they also produce high levels of VEGF in the presence of pro-inflammatory microglia (Costa, et al., 2015). Moreover, several other stromal cell-derived factors, including basic fibroblast growth factor (bFGF), angiopoietin-1, MCP-1 may support this process (Kinnaird, et al., 2004, Watt, et al., 2013). As a result, the first signs of neo-angiogenesis can be appreciated at this stage by the appearance of endothelial cell structures within the stromal cell graft (Costa, et al., 2015, Praet, et al., 2014). Fourth, at this time point a significant increase in GFAP-expression can be noticed around the graft, which implicates the start of astroglial scarring (Praet, et al., 2014). The process of reactive astrogliosis is known to be triggered by several factors, of which in the case of stromal cell implantation the most important ones are hypoxia, ATP release by damaged cells, ROS and NO production, and cytokines such as IL6, IL10, IL1, TNF α and IFN γ (Sofroniew, 2009). Altogether, our data demonstrate that the initial remodeling of stromal cell grafts in the CNS is triggered by hypoxia-mediated cell death of grafted cells, which subsequently activates neutrophils, microglia, macrophages, endothelial cells and astrocytes.

Day7: Astroglial barrier formation

During the following 3-4 days, no major changes can be observed within and surrounding the stromal cell graft, apart from an increasing number of endothelial cells, microglia and/or macrophages (Praet, et al., 2014). However, at this stage, astroglial scarring around the stromal cell graft becomes stronger (Figure 1E). Plausibly, this is

induced through a STAT3-dependent mechanism by the surviving stromal cells as well as the ongoing inflammatory processes, as a comparable situation is observed in spinal cord injury lesions where the astroglial scar surrounds inflammatory and fibrotic cells in a STAT3-dependent manner (Wanner, et al., 2013). We may assume that the observed astroglial scarring will create an effective barrier to avoid stromal cell migration on the one hand and peripheral inflammatory cell migration into the surrounding brain tissue on the other hand. From a physiological point of view, both suggestions are reasonable as stromal cells and peripheral immune cells are non-natural cells in the healthy CNS, at least in the amount present at the stromal cell graft site.

Day 10: Stabilization of the stromal cell graft

By day 10 post-implantation, the remnant stromal cells become stabilized within their new micro-environment (Figure 1F and Figure 2a). At this stage, neutrophils are no longer present and a clear distinction can be made between blood-derived macrophages mainly within the stromal cell implant and brain-resident microglia mainly surrounding the stromal cell implant, both being separated by an astroglial scar. However, we cannot rule out that a migration of microglia or macrophages through the astroglial scar occurs, since a small percentage of macrophages can be found around the astrocyte barrier. Our study demonstrating this separation, was performed in an eGFP bone marrow transplantation mouse model, in which the origin of $96 \pm 2\%$ of eGFP⁺ macrophages can be claimed as bone-marrow-derived, although we cannot exclude a minority of macrophages being derived from microglia. Nevertheless, our findings demonstrate a clear distinction between both cell types, with the astroglial scar as the visual border (Le Blon, et al., 2014). Currently we do not yet know how and why exactly this separation

of grafted stromal cells and peripheral macrophages at one end and brain-resident microglia on the other end, is established by reactive astrocytes. However, clear parallels can be drawn with natural lesion site remodeling in the CNS. For example, in a mouse model of spinal cord injury, macrophages and microglia are similarly separated by an astroglial scar, with macrophages residing within the astroglial scar and microglia surrounding the lesion site (Zhou, et al., 2014). Another study in a mouse model for TBI demonstrated that there is a temporal difference in the appearance of brain-resident microglia and infiltrating macrophages (Morganti, et al., 2015). These findings indicate that CNS resident microglia and infiltrating blood-borne macrophages contribute differently to neuro-inflammation (Jung and Schwartz, 2012, Shechter and Schwartz, 2013). Further analysis of macrophages and microglia phenotypes following stromal cell graft remodeling revealed a differential expression pattern of activation markers, like F4/80 and MHCII, on both microglia and macrophages. While graft-infiltrating macrophages express high levels of these activation markers, its expression on graft-surrounding microglia is highly reduced (Figure 2b). Consequently, this suggests that both cell types are differently activated after stromal cell grafting in the CNS. And certainly their three-dimensional separation (Figure 2c) promotes further investigation of associations between microglia and macrophage phenotype and function during stromal cell graft remodeling and furthermore the influence on, or contribution to, neuroprotection following cell grafting in the CNS.

Towards a unifying theory

In the past few years, it has become clear that stromal cell implantation into the (injured) CNS is not trivial. Indeed, assuming that soluble factors secreted by injected stromal cells and/or endogenous cell types invading the graft site will be strong enough to create a bystander effect that positively influences neuro-inflammatory and/or degenerative processes, is overly simplistic. As discussed in this manuscript, the process of stromal cell grafting, but also for other cell types like neural stem/progenitor cells (De Waele, et al., 2015, Reekmans, et al., 2012), relies on a complex interplay between hypoxia-mediated cell death of grafted cells, neutrophil invasion, microglia and macrophage recruitment, astrocyte activation and neo-angiogenesis at the graft site, ultimately leading to the survival of a limited number of grafted cells. Although the notion that only a small fraction of the initial cell graft is able to survive is well accepted, the immune-remodeling processes occurring after hypoxia-mediated apoptotic death of grafted stromal cells have been largely ignored by most studies. Comprehensibly, reports of inflammatory processes following stromal cell grafting *in vivo*, especially in the CNS, are not in favor of the current assumption that stromal cell grafting is a safe and well-tolerated procedure. However, we believe that it are exactly these inflammatory processes induced by stromal cell grafting that are of substantial importance to the overall observed neuroprotection in animal models of CNS injury. This view is supported by strong evidence in past studies demonstrating that macrophages, key players in the stromal cell graft-induced inflammatory environment, can contribute to improved disease outcome in animal models of neuropathology when activated in a ‘correct’-anti-inflammatory/neuroprotective- way (Corraliza, 2014, Hu, et

al., 2015). Therefore, it is certainly worthwhile to study not only the *in vivo* function of stromal cells, but also to reorient interest towards functional properties of stromal cell graft-associated microglia and macrophage responses. This way, stromal cell graft-induced inflammatory responses, currently considered as a harmless side effect, may turn out to be therapeutic inflammation.

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Figure legends

Figure 1. Stromal cell graft-remodeling from day 0 until day 10 after transplantation in the central nervous system.

(A) Representative image of the stromal cell graft site at the moment of transplantation. A bolus of viable stromal cells is present at the site of injection. (B) Representative image of the stromal cell graft site at 6 hours after transplantation. All stromal cells in the dense core have become hypoxic. (C) Representative image of the stromal cell graft site at 24 hours after transplantation. Nearly all hypoxic stromal cells in the core underwent apoptosis or necrosis, leaving a high concentration of stromal cell debris at the core. At this time point the graft is also infiltrated by neutrophils. (D) Representative image of the stromal cell graft site at day 3 after transplantation. The graft has become smaller with only viable stromal cells to remain. At this time point the graft becomes infiltrated by macrophages and surrounded by microglia. Furthermore, astrocytes are surrounding the implant and the first endothelial cells are appearing within the graft. (E) Representative image of the stromal cell graft site at day 7 after transplantation. At this time point more macrophages and microglia accumulate in and around the graft. Furthermore, the astroglial scar surrounding the stromal cell graft has become stronger and blood vessels are in full development. (F) Representative image of the stromal cell graft site at day 10 after transplantation. The implant is completely infiltrated by macrophages and surrounded by microglia. Meanwhile, a very strong barrier is formed around the graft by the astroglial scar. Note that at this stage, neutrophils are no longer present at the stromal cell graft site.

Figure 2. Immunofluorescent images demonstrating important cellular events during stromal cell graft-remodelling.

(A) Representative immunofluorescent images showing a blue fluorescent protein (BFP)-expressing stromal cell graft (light blue) in the CNS of CX₃CR1-eGFP x CCR2-RFP mice. Stromal cell graft surrounding microglia are visualized by direct eGFP-fluorescence (green), the infiltrating macrophages by direct RFP fluorescence (red) and the astroglial scar is visualized by immunofluorescence for GFAP (blue) at day 10 after transplantation in the CNS. (B) Representative immunofluorescent images showing the activation of infiltrating macrophages (red) and surrounding microglia (green). The two left images demonstrate F4/80 expression (blue) on macrophages and microglia, signifying a general activation state. The two right images demonstrate MHCII expression (blue) on macrophages and microglia, signifying a more specific activation phenotype. The graft is delineated by a dotted line. All above scale bars indicate 100µm. (C) 3D recording of a cleared CX₃CR1-eGFP x CCR2-RFP brain tissue block (1273 µm² x 500 µm) containing the stromal cell graft illustrates the invasion of macrophages (Ma) in the center of the graft site, while microglia (Mi) are only observed surrounding the graft. The injection-tract shows a similar invasion pattern, with macrophages inside the tract, and microglia encapsulating it. A 2D (XY-XZ, left) and 3D (right) orthogonal view are shown.

FIGURE 1

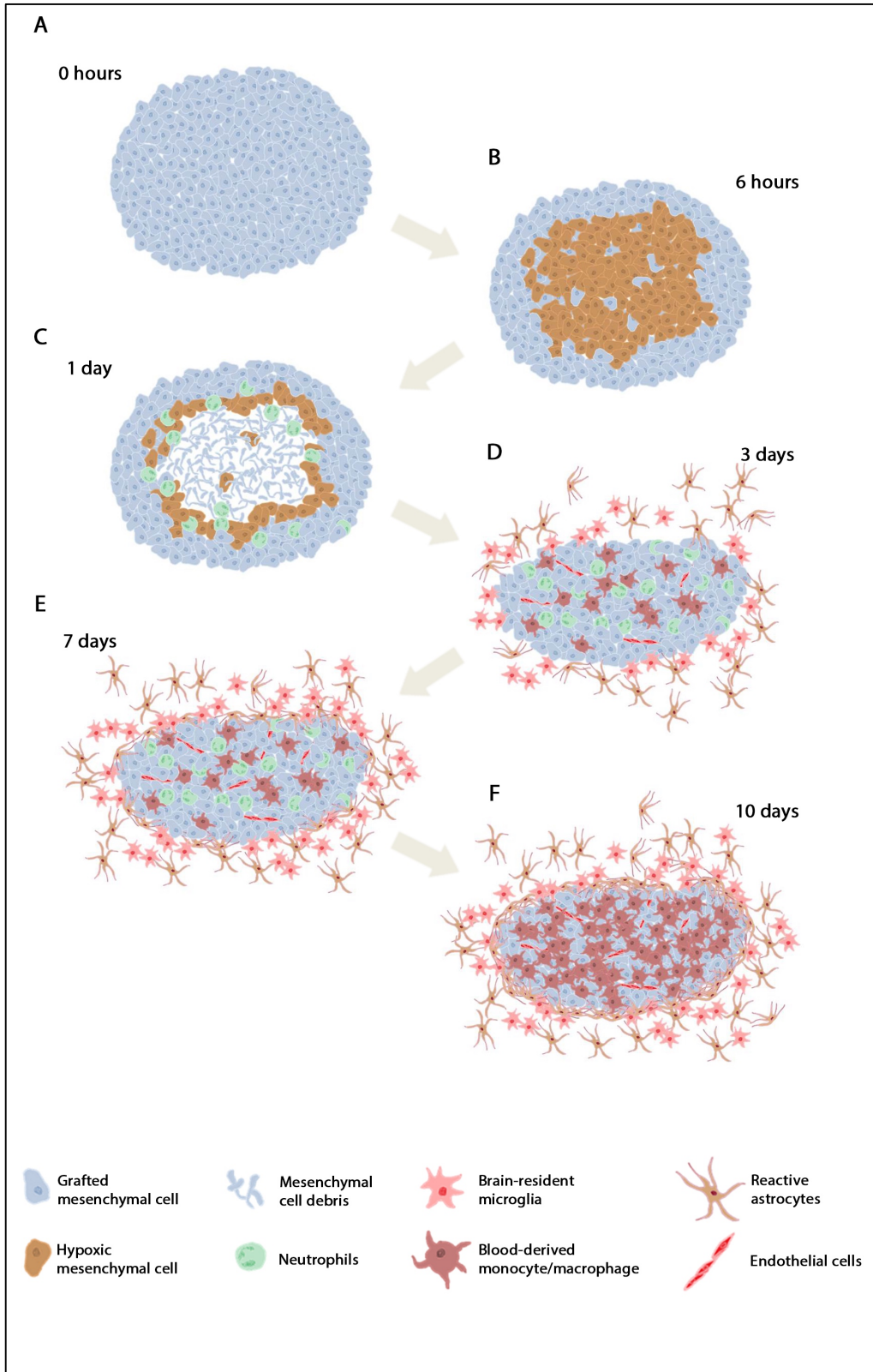


FIGURE 2

