

Immunoregulatory Neuroprotection of Cerebral Ischaemia by Haematopoietic Stem and Precursor Cells

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Abstract

Cerebral ischaemia leads to early immune system activation followed by delayed immunosuppression. Post-ischaemic inflammation contributes to neurodegeneration. Although experimental approaches using adult stem or precursor cells have repeatedly demonstrated neuroprotective effects in cerebral ischaemia, the underlying mechanism of cell-mediated neuroprotection is still debated. It was suggested that stem or precursor cells invade ischaemic brain regions and act locally. However, recent data demonstrate that systemically transplanted stem or precursor cells have strong immunoregulatory effects leading to reduced post-ischaemic brain tissue inflammation. This article argues that the systemic balance of the immune system might explain the reduced neurodegeneration observed after stem cell treatment in cerebral ischaemia. Consequently, systemic immunoregulatory neuroprotection using stem and precursor cells should be considered an important therapeutic option to prevent post-ischaemic neurodegeneration in cerebral ischaemia.

Keywords

Stroke, cerebral ischaemia, neuroinflammation, neuroimmunology, neuroprotection, neurodegeneration, haematopoietic stem cells, haematopoietic precursor cells, stem cell therapy

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Evidence-based stroke therapy using the thrombolytic agent recombinant-tissue plasminogen activator (rt-PA) aims at rapid recanalisation of the occluded vessel within the acute phase of cerebral ischaemia, thereby lacking direct neuroprotective or neuroregenerative properties to restrict subsequent ischaemic damage.¹ In the search for promising neuroregenerative tools, stem cell treatment has come into the spotlight during the last decade of experimental stroke research. Since then, various kinds of stem and progenitor cell from different kinds of tissue have been exploited for their neurorestorative and neuroregenerative properties and value in cerebral ischaemia.²⁻⁵

Despite all efforts, none of these studies has convincingly achieved the ultimate goal: stem cell differentiation into neuronal phenotypes accompanied by functional integration into neuronal networks. However, evidence of graft-cell-mediated neuroprotection in stroke has emerged in many studies.⁶⁻⁸ Skepticism remained as most of the data failed to convincingly demonstrate *in vivo* neuronal transdifferentiation. Consequently, other mechanisms were propagated, such as stimulation of endogenous neurogenesis via neoangiogenesis in peri-infarct tissues.⁹ Surprisingly, neuroprotection following cell treatment after experimental stroke was observed despite the absence of central nervous system invasion of exogenous stem cells. Thus, functional recovery was suggested to be due to graft cell release of soluble mediators that cross the post-ischaemic blood-brain barrier and act on the lesion site.¹⁰

Indeed, subsequent studies detected most of the grafted cells primarily in secondary immune organs, such as the spleen and lymph nodes.¹¹ The authors' experiments confirmed early and numerous detection of systemically injected green-fluorescent-protein-positive haematopoietic stem cells (HSCs) in the spleen. This was followed by a delayed and limited cell migration into ischaemic brain parenchyma and significant neuroprotection after focal cerebral ischaemia in mice.¹² However, the authors' data suggest that systemic immunomodulatory mechanisms are responsible for the substantial neuroprotection observed after stem cell treatment in stroke experiments.

This review re-visits recent studies on post-ischaemic inflammatory neurodegeneration, involvement of the peripheral immune system and interference by transplantation of stem and precursor cells in cerebral ischaemia.

Neuroprotection in Ischaemia

Sudden blockage of the oxygen and glucose supply in cerebral ischaemia initiates various cascades of pathophysiological events terminating in neuronal cell death.¹³ Within the ischaemic penumbra tissue, aggressive processes take place including excessive activation of glutamate receptors, accumulation of intracellular calcium cations, recruitment of inflammatory cells and excessive production of free radicals.^{14,15} Such secondary autodestructive reactions, which are activated over a period from seconds to days after the primary insult, cause an exacerbation of cell death.¹³

Most of the studies on stroke pathophysiology have explored treatments that interrupt propagation of these cascades in order to achieve brain tissue protection. Several treatments interfering with these pathophysiological cascades have been tested in experimental stroke and have been shown to be neuroprotective.¹⁶ However, none of the neuroprotective approaches tested in clinical trials so far has convincingly demonstrated functional benefits.¹⁷

Post-ischaemic Inflammation and Neurodegeneration

Cell damage progression leading to further neurodegeneration early after the onset of cerebral ischaemia is mainly attributed to inflammatory events.^{18,19} Following ischaemic brain injury, early activation of transcription factors (e.g. nuclear factor- κ B) is found locally in brain cells, leading to an upregulation of pro-inflammatory cytokines and chemokines,²⁰⁻²³ including interleukin-1-beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α). These factors are secreted by activated microglial cells, astrocytes,²⁴ endothelial cells and neurons,^{18,22} as well as by infiltrating mononuclear cells from blood.²⁵

Recruitment of peripheral immune cells from the blood and spleen to the site of brain injury via a compromised blood-brain barrier is mediated by different chemotactic factors.^{26,27} Chemokines also play a role in mobilisation and attraction of bone marrow-derived stem and precursor cells towards brain lesions.²⁸⁻³⁰ Due to increased expression of vascular adhesion molecules on endothelial cells, several immune cell types including blood neutrophils (within ~48 hours), monocytes, macrophages (within ~18 hours) and T cells (within ~72 hours) infiltrate the brain tissue and exacerbate the ongoing tissue damage.^{27,31} Subsets of immune cells attracted to the lesion site, namely activated microglia and invaded macrophages, have primarily beneficial functions, such as clearance of debris.³² However, cellular release of cytotoxic molecules including inflammatory cytokines (e.g. TNF- α), complement factors and free radicals has a destructive effect on tissue.³³

By contrast, the role of T cells found close to blood vessels in peri-infarct areas 24 hours after reperfusion is less clear.^{26,34} Brain-specific T cells, as part of the adaptive arm of the immune system, might attack the brain tissue and exacerbate the damage.³⁵ A large increase in splenic T-cell cytokine and chemokine production occurs early after reperfusion and is suggestive of a role in the adaptive immune response to stroke.²⁵

Early Peripheral Immune Activation

Focal cerebral ischaemia not only produces local inflammatory events within the ischaemic brain and attracts peripheral immune cells to the lesion site, but also exerts long-distance effects on lymphoid organs by modulating the function of the spleen.³⁶ It is unclear how the brain communicates a 'danger signal' to the spleen. One underlying mechanism proposed is sympathetic nervous system activation. Ischaemic brain tissue damage results in the release of pro-inflammatory cytokines (e.g. TNF- α) and chemokines from splenocytes in the bloodstream.^{25,37-39}

Within the first 24 hours following cerebral ischaemia, spleen cells release significant amounts of pro-inflammatory (TNF- α , IFN- γ , IL-6, MCP-1 and IL-2) and anti-inflammatory (e.g. IL-10) cytokines. This is followed by extensive movement of leukocytes from the spleen and thymus.⁴⁰ The leukocytes appear to spread into the blood and invade the ischaemic penumbra.^{36,39}

Removal of the spleen before experimental stroke was performed by Ajmo et al. to evaluate the role of the peripheral immune system in cerebral ischaemia. Ablation of the largest pool of immune cells (macrophages, neutrophils, B cells and T cells) due to splenectomy resulted in smaller infarct size and attenuated neuroinflammation after permanent middle cerebral artery occlusion (MCAO).⁴¹ Immunohistochemically, brain infiltration by activated microglia, macrophages and neutrophils was reduced in the ischaemic hemispheres of splenectomised rats compared with controls.⁴¹ From this experiment a splenic origin of neutrophils infiltrating post-ischaemic brain tissues was assumed since blood leukocyte counts did not differ between the groups. Even when normalised to infarct volume, animals that had been splenectomised before showed less neutrophil infiltration and reduced brain tissue damage following MCAO.⁴¹

To further analyse the role of lymphocytes in post-ischaemic inflammation, experimental stroke was performed using severe combined immunodeficiency (SCID) mice lacking T and B cells.⁴² Naive SCID mice displayed a drastically diminished number of splenocytes, as expected in the absence of T and B cells. MCAO further reduced the cell number per spleen. However, the number of blood mononuclear cells remained unchanged after ischaemia. In comparison with wild-type animals, SCID mice were robustly protected from ischaemic injury. Both cortical and total infarct volumes were reduced in SCID mice, suggesting that T and B lymphocytes are harmful players in early ischaemic brain injury. However, protection of the ischaemic core was not achieved by the absence of T and B cells.⁴²

Delayed Peripheral Immunodepression

Cerebral stroke was repeatedly shown to result in post-ischaemic atrophy of the spleen and thymus, as well as in reduced splenic and thymic function.^{36,40,43} Systemic immunosuppression within days after focal stroke has been widely observed and presumably contributes to microbial infections associated with stroke.^{44,45} As mentioned above, sympathetic nervous system activation due to brain injury was proposed to lead to rapid and severe functional alteration of the immune system, resulting in a stroke-induced immune depression syndrome (SIDS).^{43,46,47} SIDS is manifested by reduced T-cell activation and a profound loss of immune T and B cells in the blood, spleen and thymus.⁴³ Alterations in splenic function are either due to an increase in splenocytic cell death or to a shift in distribution of splenocyte subtypes. Sympathetic signalling to the spleen and thymus stimulates an overabundance of CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells, which might inhibit protective immunity. These so-called 'master regulators' of the immune system normally limit inflammation and inhibit autoimmune diseases.⁴⁸⁻⁵¹

In summary, inflammatory mechanisms following stroke result in a biphasic response of the immune system. Initially, rapid activation of splenic T lymphocytes is accompanied by a release of cytokines within the first hours after stroke, propagating local inflammatory processes within the damaged brain. Then, in the subacute phase of ischaemia, extended and progressive cell death of splenocytes is induced, leading to massive loss or redistribution of the remaining immune cells.^{40,43} Such an exhaustion of immunocompetent cells results in an inability to adequately respond to microbial challenges due to systemic immunosuppression.⁴⁷

Stem and Precursor Cell Sources

The adult bone marrow consists of several distinct populations of stem cells. HSCs and endothelial progenitor cells are offspring of the haemangioblast cells that are believed to be among the most primitive of all cells in bone marrow.⁵² HSCs are multipotent, and their direct progeny, haematopoietic precursor cells, are the immediate ancestors of all mature blood cells.⁵³ Apart from bone marrow, HSCs can be obtained from peripheral blood,⁵⁴ umbilical cord⁵⁵ and foetal liver.⁵⁶

Overall, circulating HSCs and haematopoietic precursor cells are rare, comprising only 1/10,000 to 1/100,000 of total blood cells. They make up only a small fraction of total CD34⁺ cells isolated from haematopoietic tissue sources.⁵⁷ The majority of CD34⁺ or lineage-depleted cells consist of lineage-committed progenitor cells. Human haematopoietic stem cells are lineage-negative. They lack expression of surface markers including CD33, HLA-DR and CD45RA, but they do co-express the specific markers Thy-1, c-Kit and CD133.^{58,59} In mice, HSCs have been identified as lineage-marker-negative (Lin-), Sca-1⁺ and, optionally, c-Kit⁺ cells. Expression of CD34 on mouse HSCs depends on the mouse strain, the developmental stage and the status of activation of these cells.⁶⁰ Compared with bone-marrow-derived CD34⁺ lineage-negative HSCs, the proliferation and differentiation capacity of mobilised peripheral blood CD34⁺ lineage-negative cells is inferior.⁶¹

In experimental studies, Weissmann et al. showed that single HSCs reconstituted peripheral blood leukocytes but did not contribute to non-haematopoietic tissues, including brain, kidney, gut, liver and muscle. This suggests that transdifferentiation of circulating HSCs or their progeny is an extremely rare event, if it occurs at all.⁶²

The process of homing and engraftment of bone-marrow-derived stem cells is attributed to the surface chemokine receptor CXCR4, expressed on bone marrow stem cells. It is also attributed to the signalling chemokine stromal-derived factor-1, which is produced by bone-marrow-associated sinusoidal cells, but also in damaged tissue, including cerebral ischaemia.⁶³

Umbilical cord blood is another rich source of HSCs and is thus an interesting alternative to bone marrow due to its broader availability, weak immunogenicity and lower risk of viral contamination.⁶⁴ Umbilical cord blood stem cells (UCBCs), accounting for 2% of human umbilical cord blood, are capable of reconstituting all blood lineages. In addition, when exposed to selective growth factors, UCBCs can create progeny that obtain certain properties of neuronal and glial cells.⁶⁵

Immunomodulatory Stem Cell Therapy

Brain tissue infiltration by immune cells, including monocytes and neutrophils, is highly indicative of an ongoing inflammatory response early after stroke. In addition, haematopoietic stem and precursor cells have been shown to be recruited towards the ischaemic brain lesion in mice, suggesting a modulatory impact on cerebral ischaemia.^{66,67}

Intravenous administration of human UCBCs in the rat following experimental stroke resulted in significant behavioural benefits and was superior to intrastriatal transplantation.⁶⁸ The human UCBCs secreted numerous angiogenic factors, including vascular endothelial growth factor, hepatocyte growth factor and insulin-like growth factor-1.⁶⁹

Additionally, human UCBCs were shown to influence immune cell infiltration into the injured brain after experimental stroke and were associated with reduction of infarct volume and improved functional outcome.¹¹ The grafted human UCBCs migrated into the spleen, indicating a splenic impact of these cells during the acute and subacute phases of stroke.¹¹

Although a decrease in CD45⁺/CD11b⁺ microglia and macrophages and CD45⁺/B220⁺ B cells was found, neither CD45⁺/CD3⁺ T cells nor neutrophil cell counts changed in the infarcted hemispheres of UCBC-treated humans compared with controls.¹¹ Additionally, decreased messenger RNA (mRNA) expression of TNF- α and IL-2 reduced protein expression of TNF- γ and IL-1 β . Decreased mRNA also had a tendency to increase IL-10 (an anti-inflammatory cytokine) expression in animals receiving human UCBC stem cell treatment.⁷⁰ *In vitro*, human UCBCs were shown to act in a neuroprotective manner that was referred through the release of diffusible factors.^{10,71} *In vivo* human UCBCs administered 24 hours after MCAO inhibited the inflammatory response; however, the administration significantly increased the level of IL-10.⁷⁰

Spleen weight reduction observed after permanent MCAO was reverted in human UCBC-treated animals. Spleens from MCAO rats showed significantly reduced numbers of CD8⁺ T cells compared with stem cell-treated animals.⁷² While the splenic cell number of CD4⁺ T cells did not differ between groups, MCAO-induced reduction in depression of the CD8⁺/CD4⁺ ratio was almost normalised in the stem-cell-treatment group.⁷² *In vitro* experiments showed that human UCBC treatment resulted in inhibited T-cell response to mitogens normally observed after experimental stroke. Whereas mRNA expression of the pro-inflammatory cytokines TNF- α and IFN- γ was reduced, expression of IL-10 was elevated after human UCBC delivery. Vendrame et al. hypothesised that human UCBC treatment inhibited MCAO-associated T-cell activation due to modulating the expression levels of pro-inflammatory cytokine IFN- γ and anti-inflammatory cytokine IL-10, resulting in attenuated ischaemic brain injury.⁷²

The authors' study on lineage-negative HSC administration in experimental MCAO in mice showed early detection of HSCs in the spleen and later migration of green-fluorescent-protein-positive cells into the peri-infarct brain parenchyma.¹² Although a significant reduction in infarct size and apoptotic neuronal cell death was observed in brains of HSC-treated animals, realtime polymerase chain reaction (PCR) analysis showed no significant production of neurotrophic factors or cytokines by lineage-negative HSCs *in vitro* or *in vivo*. Instead, significantly reduced central nervous system infiltration of peripheral immune cells, namely T cells and macrophages, in peri-infarct brain tissues of stem-cell-treated mice was found.¹²

In line with this, realtime PCR analysis of different cytokine and chemokine receptor levels in the spleens of mice that underwent MCAO before revealed that HSC mediated downregulation of proinflammatory cytokines and chemokine receptors. This resulted in inhibited activation of immune gene transcripts in splenocytes.

In summary, attenuated peripheral post-ischaemic immune response by HSC treatment in mice was demonstrated, leading to reduced immune cell infiltration into ischaemic hemispheres accompanied by significant neuroprotection after experimental MCAO.¹²

Conclusion

Cerebral ischaemia first is a local event in the brain, but has strong secondary systemic effects on the whole organism. The attenuation of the peripheral immune response after ischaemia has been underestimated for many years. Cerebral ischaemia induces strong immune activation in the spleen followed by delayed immunosuppression. Within 24 hours after cerebral ischaemia, splenocytes upregulate chemokine receptors and pro-inflammatory cytokines, which support their recruitment to the ischaemic brain lesion. Post-ischaemic neurodegeneration is partly mediated by these recruited immune cells. Data from stem and precursor cell transplantation experiments show strong immunoregulatory activity and interference with the early post-ischaemic immune activation of the spleen. Transplanted stem and precursor cells are detected substantially within the spleen. They attenuate splenic pro-inflammatory activity and chemokine receptor expression after cerebral ischaemia. Consequently, due to stem and precursor cell treatment, a lower number of immune cells become recruited to the ischaemic lesion site and post-ischemic inflammation is

reduced, resulting in neuroprotection. The response of the immune system – peripheral and central immune activation followed by peripheral immunosuppression – should be considered an important target in stroke therapy. ■



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