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Treating Cerebral Ischemia: Novel Therapeutic Strategies from Experimental Stroke Research

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Abstract: Although systemic thrombolysis and endovascular treatment have revolutionized modern stroke treatment, the majority of patients do not qualify for either treatment paradigm. Hence, novel adjuvant therapeutic strategies are required. This chapter provides an overview of our current understanding of novel therapeutic strategies in preclinical stroke models. The chapter is organized in three major parts to cover the acute, subacute, and chronic phases of ischemic stroke. The potential of various pharmacological agents, stem cells, microRNAs, and extracellular vesicles as therapeutic avenues along with the progress and challenges are discussed.

Keywords: cerebral ischemia; extracellular vesicles; neuroprotection; neurodegeneration; stroke

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INTRODUCTION

The temporal patterns of stroke are divided into acute, subacute and chronic stages of the disease (1). The diagnostic procedures for patients with clinical signs of an acute ischemic stroke include brain imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) (2). Beside these well-established imaging tools, a variety of novel stroke biomarkers including proteins, lipids, RNAs, and metabolites are subject to current research (3, 4). However, none of them has been translated into routine clinical settings, yet. Stroke is an emergency, and time is a crucial factor for not only diagnostic but also therapeutic procedures. Causal treatments of acute ischemic stroke include systemic thrombolysis and mechanic thrombectomy (5), which are reserved for a minority of patients because of contraindications and narrow time windows. In addition, revascularization itself does not halt the stroke-induced activation of proinjurious signaling cascades within the ischemic tissue. The availability of novel adjuvant neuroprotective tools may therefore lead to a paradigm shift in stroke therapy. Numerous preclinical studies with potential therapeutic agents that modulate the pathophysiological process in the acute, subacute, and chronic phases of ischemic stroke have been conducted. However, despite extensive research, translation from preclinical findings to clinical routine has not yet been successful.

ACUTE PHASE OF STROKE

For the acute phase of stroke, research activities have predominantly focused on neuroprotection. Many pharmacologically active drugs have been found to be neuroprotective in preclinical stroke settings (6). Such pharmacological interventions target various aspects of the complex pathophysiology of stroke, among which are excitotoxicity, oxidative stress, calcium influx, programmed cell death, immune system response, and blood-brain barrier (BBB) disruption (7). Although translation of promising preclinical data into the clinic has failed thus far (8), promising research activities continue to occur worldwide. Some of these novel treatment strategies are discussed in the following paragraphs (Figure 1).

Glutamate-mediated excitotoxicity, which involves N-Methyl-D-Aspartat receptors (NMDAR), has been considered as one of the most important cell death mechanisms in the pathogenesis of stroke (9). Several drugs, particularly inhibiting NMDAR, were found to be neuroprotective in preclinical studies (10). Beside directly inhibiting NMDAR, some studies focus on analyzing downstream targets (11). For instance, the PSD95 inhibitor Tat-NR2B9c (NA-1) has shown neuroprotective effects by decreasing the ability of NMDAR to trigger downstream excitotoxicity via neuronal nitric oxide synthase (nNOS) activation (11).

Ischemia-induced oxidative stress leads to vasodilatation and altered vascular reactivity which in turn promote BBB breakdown and severe tissue injury (12). Oxidative stress includes the generation of superoxides and nitric oxides, which are both linked to excitotoxicity (13, 14). Free radical scavengers like edaravone, uric acid, and citicoline have been identified as potential neuroprotective agents (15). Excessive Ca^{2+}-influx leading to toxic intracellular levels is a major factor for triggering detrimental ischemic signal cascades, such as the activation of

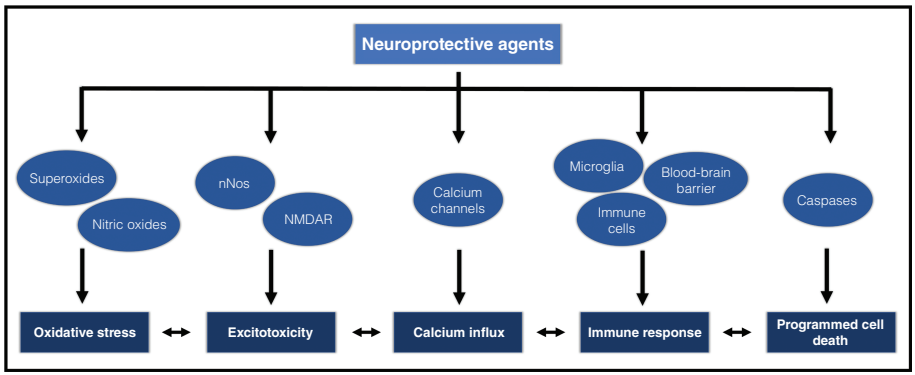

Figure 1. Main targets in acute phase of ischemic stroke by neuroprotective agents. Preclinical neuroprotective candidates mainly target one or more of various key players during the acute stage of ischemic stroke. These targets include oxidative stress, excitotoxicity, calcium influx, immune response and programmed cell death, all of which are tightly interconnected. Examples of particular downstream mechanisms are shown. Abbreviations: nNOS, neuronal nitric oxide synthase; NMDAR, N-Methyl-D-aspartic acid receptor.

proapoptotic enzymes and synthesis of free radicals (7). Thus, pharmacological inhibition of Ca^{2+} overload is a major field in neuroprotective stroke research. There are two major strategies: blocking of calcium channels, and modulation of calcium-related signaling molecules. Calcium channel blockers (CCB) such as lercanidipine and cilnidipine lead to neuroprotection by attenuating oxidative stress, inflammation and apoptosis (16, 17). Furthermore, intraarterial delivery of Verapamil, a routinely used CCB in the treatment of cardiac diseases, leads to neuroprotection in experimental models of stroke (18). A promising candidate for regulating the calcium-related downstream molecules is paeoniflorin. Paeoniflorin has been shown to decrease neurological deficit scores and infarct volumes in rats by regulating the Ca^{2+}/calmodulin-dependent protein kinase II/response element-binding signaling pathway (19).

Ischemia-induced changes in cellular homeostasis trigger programmed cell death such as apoptosis, necroptosis, and autophagy. The cell death mechanism of each single cell is mainly determined by the extracellular milieu, cell type, cell age, and location in the brain (20). It is worth noting that in the last decades the view on programmed cell death has changed, since additional forms and hybrid forms were discovered (21). Modulating these ischemia-induced cell death signal cascades is one of the major experimental approaches to reduce neuronal cell loss after ischemic stroke. Caspase inhibitors, for example the caspase-3 inhibitor z-DEVD-fm, effectively inhibit apoptosis and lead to neuroprotection in rodent models (22, 23). On the contrary, necroptosis has been found to have caspase-independent regulation pathways (24). Pharmacological inhibition of the latter using receptor-interacting serine/threonine-protein kinase (RIP) inhibitors lead to a reduction of the lesion size in mice after experimental stroke (25). The precise role of autophagy as the third main mechanism of programmed cell death after ischemic stroke is not fully understood and remains controversial (26). However, cumulative evidence from preclinical in vitro and in vivo studies suggests a neuroprotective effect of pharmacological autophagy inhibition (27–29).

Acute poststroke inflammation and stabilization of the BBB

Acute neuroinflammation after ischemic stroke is a highly complex sequence of events. In the following, we focus on three main factors and their role as targets in neuroprotective strategies, i.e., the activation of resident microglia, the opening of the BBB, and immune cell infiltration. Although microglia have been a subject of research for quite some time, the precise role of microglia under ischemic conditions is not yet known. Microglia are involved in both promoting ischemic brain damage and facilitating poststroke recovery. The latter depends on the state of function as expressed by microglia polarization (30). Thus, pharmacological modulation of microglial polarization is a promising neuroprotective strategy. For example, fingolimod, currently used in the treatment of multiple sclerosis, has been shown to modulate microglial polarization, leading to acute neuroprotection and enhanced angiogenesis in rodent stroke models (31, 32).

Inhibiting stroke-induced BBB breakdown, which plays a detrimental role in neuroinflammation and in the development of neurological dysfunctions, was identified as a possible therapeutic strategy (33). The main strategy for pharmacological stabilization of the BBB is the inhibition of matrix metalloproteases (MMP) which are responsible for the degradation of BBB components (34). Several preclinical neuroprotective agents such as resistin, lithium, and apelin-13 have been shown to stabilize the BBB as part of their neuroprotective mechanisms (35–37). BBB breakdown is one important factor for the infiltration of peripheral immune cells, including neutrophils, T cells and B cells, as well as neutrophil-derived reactive oxygen species, cytokines, and proteases; they promote early neuroinflammation. Thus, neutrophils are examined as treatment targets by blocking neutrophil adhesion to endothelial cells, thus inhibiting the infiltration into the brain parenchyma (38). However, despite promising preclinical results by using adhesion blockers such as enlimomab, an anti-intercellular adhesion molecule 1 (ICAM-1) antibody, the clinical translation has failed (39). T cells were shown to have dual roles in stroke pathophysiology depending on their subtype. While $CD4^+$, $CD8^+$, and γδT cells seem to promote brain injury, T_{reg} cells (regulatory T cells) have been shown to have neuroprotective effects (40, 41). Consistently, amplification of T_{reg} cells by CD28SA leads to a reduction in infarct size in mouse model of stroke (42). In analogy to regulating T cells, B_{reg} (regulatory B cells) are also involved in the poststroke inflammatory response (43). Again, the increase of B_{reg} cells, with special emphasis on interleukin-10 producing subtypes, revealed protective effects in preclinical stroke models (44).

Transplantation of stem cells and delivery of extracellular vesicles (EVs)

Beside neuroprotective drugs, stem cell transplantation and stem cell-derived EVs have gained increasing interest in recent years. Several types of stem cells such as neural progenitor cells (NPC) and mesenchymal stem cells (MSC) have been tested in animal models of stroke, achieving promising beneficial effects (45). Stem cell transplantation has been proven safe for patients during the acute phase of stroke (46). Similar to pharmacological intervention targets, stem cell transplantation also affects several signaling pathways, such as BBB disruption and

immune system response (47, 48). For instance, transplanting NPC into mice exposed to middle cerebral artery occlusion (MCAO) within 24 hours reduces infarct size and decreases BBB damage (49). After NPC transplantation, Evans blue dye and IgG leakage into the brain parenchyma are significantly reduced, suggesting BBB-stabilizing effects of NPC (49, 50). The BBB-stabilizing results from NPC are mainly achieved by inhibiting MMP activation and regulation of microglia activation (49, 50). Transplanted NPC can also affect peripheral immune organs, leading to a decrease of inflammatory cytokines such as TNF-α, IL-1β, IL-6, and Iba-1 (51). These beneficial effects are not limited to NPCs. MSCs, for instance, can also achieve the same effects (51). In contrast to common initial belief, however, the majority of transplanted stem cells are trapped in peripheral organs, suggesting that grafted stem cells stabilize the BBB and promote neural regeneration by indirect means (52). Such indirect means include paracrine factors. The latter became obvious when conditioned medium from stem cells was found to be as therapeutically effective as stem cells themselves (53–55).

Whereas initial work claimed soluble factors to be the biological mediators of the aforementioned effects, recent work has resulted in the concept of EVs (56, 57). The latter are a heterogeneous group of vesicles in the nanometer range that include exosomes and microvesicles, to name a few (58). In this context, EVs are capable of transporting a wide variety of molecules and mediators like proteins (e.g., cell adhesion molecules, signal molecules, membrane organizing proteins), nucleic acids (e.g., microRNAs-[miRs]), as well as lipids (59). EVs are critically involved in intercellular communication under both physiological and pathophysiological conditions, supporting cell protection, cell regeneration, as well as immune modulation (57, 59). The application of stem cell-derived EVs therefore offers a great opportunity for stroke treatment. Moreover, EV administration appears to be safe in mammals, including humans, thus avoiding potential side effects of stem cell transplantation, in particular the risk of malignant stem cell transformation (57, 60).

Preclinical research has shown that the therapeutic potential of EVs is not inferior to stem cell transplantation (61–64). Compared to stem cells, stem cell-derived EVs are easier to obtain and face fewer ethical issues. Such EVs affect multiple stroke-associated aspects, including angiogenesis, neurogenesis, and neuroprotection (65). Recent evidence suggests that miRs inside EVs are key players of EV-induced neuroprotection (66). Among these, miR-21a, miR-26a, and miR-126 are associated with strong and robust effects in preclinical stroke models, as they explicitly reduce infarct volume and stimulate neurogenesis as well as angiogenesis (67–69).

Nevertheless, both stem cell transplantation and EV delivery under stroke conditions predominantly focus on subacute or even chronic stages of the disease. Hence, only a few studies analyzed the impact of stem cells during the acute phase of stroke. A phase II clinical trial (MASTERS, NCT01436487) by Hess and colleagues on patients with acute ischemic stroke, however, revealed no significant difference in global stroke recovery after intravenous treatment with multipotent progenitor cells between 24 and 48 hours after ischemic stroke (46). This is followed by a phase III clinical trial (MASTERS-2, NCT03545607) which investigates treatment with adult stem cells given intravenously 18-36 hours after stroke (MASTERS-2, NCT03545607). For the latter, results are not available yet. The therapeutic potential of stem cells and EVs alike are further discussed in the paragraph on chronic stroke.

SUBACUTE PHASE OF STROKE

In the acute phase of stroke, cell death, inflammation and scarring processes start within minutes to hours after stroke onset. The subacute phase is characterized by tissue reorganization. However, this process is limited by decreasing endogenous plasticity of the lesioned brain tissue. Whereas stroke leads to oxidative stress, excitotoxicity, reperfusion injury, and inflammation, poststroke recovery requires the stimulation of neuronal circuits and the induction of molecular growth programs in the brain (70). Interestingly, axonal sprouting, neurogenesis, gliogenesis and angiogenesis share common features like structural growth as well as interactions with other cells. However, these molecular mechanisms only represent transient regenerative cellular niches for neural repair after stroke (70). Li and colleagues report a significantly reduced gene regulation of the axonal sprouting connectome three weeks after stroke, indicating the loss of a coordinated growth state and a molecular closure of the sensitive period in the subacute phase after stroke (71). The global goal of stroke treatment therefore is to increase either the amount or the duration of endogenous plasticity.

Immunological aspects

The acute effects of ischemic stroke also lead to the activation of microglia and astrocytes which secrete proinflammatory factors, further recruiting peripheral immune cells to the ischemic area (72, 73). Additionally, dying neurons themselves, astrocytes, and endothelial cells release danger-associated molecular patterns (DAMPs), which activate microglia as well as peripheral immune cells in a detrimental cascade (72, 73).

Under physiological conditions, microglia are the innate immune cells of the brain, in their resting state constantly surveilling their environment and contributing to tissue homeostasis (74). Ischemic injury initiates the modulation of the resting microglia to an activated state as an immediate response. In the acute phase after stroke, activated microglia mainly represent the M1 type, inducing proinflammatory effects with detrimental consequences for the neurons in the ischemic penumbra (75, 76). In the subacute phase of stroke, however, the microglia population further shifts to the M2 type with an antiinflammatory purpose (77). This M1/M2 shift is often referred to as a double-edged sword action of microglia during stroke, which can be both beneficial and detrimental. Recent transcriptomic analyses of activated microglia display an activation of pathways outside the classic M1/M2 polarization paradigm, making further invesigations regarding this heterogeneity recommendable (74).

After the initial accumulation of microglia, other immune cells such as macrophages, lymphocytes, dendritic cells, and neutrophils infiltrate the lesion site (78). The latter are the main cause for the BBB breakdown after stroke (38). Whereas some studies claim that microglia overall seem to have largely protective effects after stroke, T lymphocytes of the innate immune system are regarded to be highly detrimental (79). On the other hand, T_{regs} of the adaptive immune system are thought to have protective effects. T_{regs} invade the ischemic tissue after the acute phase of stroke and act as neuroprotectors via the secretion of IL-10 (78). However, the exact mode of function of T_{regs} after stroke needs to be further elucidated.

B lymphocytes have also been described to have both detrimental and protective properties after stroke (79). Doyle and collegues report that B cells can be detected in the affected brain tissue in mouse models of stroke as well as in human patients (80). The authors link this finding to the later development of dementia after stroke (80). Protective effects are rather mediated by B_{regs} and, in accordance to T-cells, the secretion of IL-10 plays a major role against inflammation and resulting neurologic deficits (81–83). IL-10 inhibits a variety of T-cell proinflammatory responses and increases the population of T_{regs}. Furthermore, latest research discovered additional molecules secreted by B_{regs} to regulate immune responses, like TGF-β (transforming growth factor beta), IL-35 and granzyme B, as well as surface proteins like CD1d and PD-L1 (84). However, more studies are needed to unravel these novel pathways, as the understanding of B_{regs} function after stroke appears to be a promising treatment strategy for transfer into the clinics. However, the dual role of inflammation after ischemic stroke, affecting both injury and repair, remains challenging, and therapeutic approaches targeting several cell types at different time points after stroke appear most promising (72). An overview of the different immune cells being involved in the pathology of stroke is given in Figure 2.

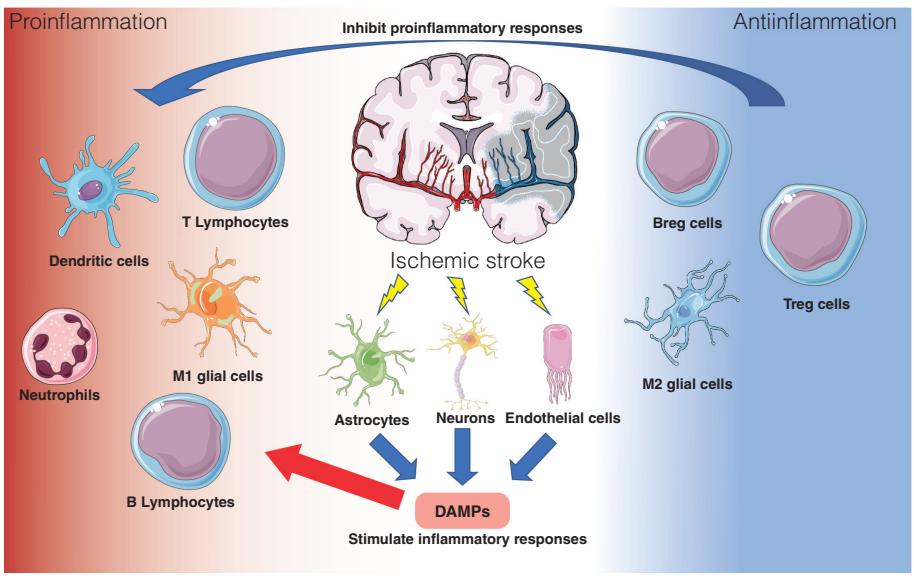

Figure 2. An overview of the different immune cells involved in the pathology of stroke. Neurons, astrocytes and endothelial cells release danger-associated molecular patterns (DAMPs) after stroke. DAMPs activate microglia and peripheral immune cells such as T cells and B cells, yielding a proinflammatory cytokine secretion profile from these cells. Meanwhile M2 microglia, regulatory T cells (Treg) and regulatory B cells (Breg) release antiinflammatory mediators that help reduce neuroinflammation. However, the dual role of inflammation after ischemic stroke is still unclear.

Gut-brain-axis

One of the largest compartments for immune cells in the body is the gastrointestinal tract (79). Here, commensal gut bacteria have direct contact to the intestinal epithelium as well as intestinal immune cells, supporting the host immune system with regard to peripheral immune education and homeostasis (79, 85, 86). The vital importance of these interactions, providing an intact immune system, was described more than half a century ago (87). More recently, the interactions between the gut microbiome and the immune system have been investigated with respect to stroke. Rosser and co-workers report that a disturbed gut microbiome negatively affects B_{regs} differentiation (88). Benakis and colleagues show that a disturbation of the gut microbiome by antibiotic treatment in the MCAO mouse model leads to an increase of T_{regs} and a simultaneous reduction of γδT-cells, correlating to decreased infarct size and increased behavioral outcome (89). However, a complete deletion of the gut microbiome had deleterious effects with regard to survival in the same model (90), pointing towards a sophisticated balance of the gut microbiome to provide beneficial effects. Furthermore, stroke itself has been shown to affect the gut microbiome composition via increased sympathetic activity, decreasing the intestinal motility, inducing dysbiosys and by that reducing microbial diversity (91). Taken together, the aforementioned studies indicate the existence of a bi-directional gut-brain-axis—an interaction of the gut microbiome via the intestinal immune system with the ischemic brain (Figure 3). After brain injury, the gut microbiome is altered and in turn modulates stroke outcome via modulation of postischemic inflammatory responses (92). Currently, the first clinical trials are conducted to further investigate how dysbiosis of the gut microbiome may influence immune response and outcome after stroke (93).

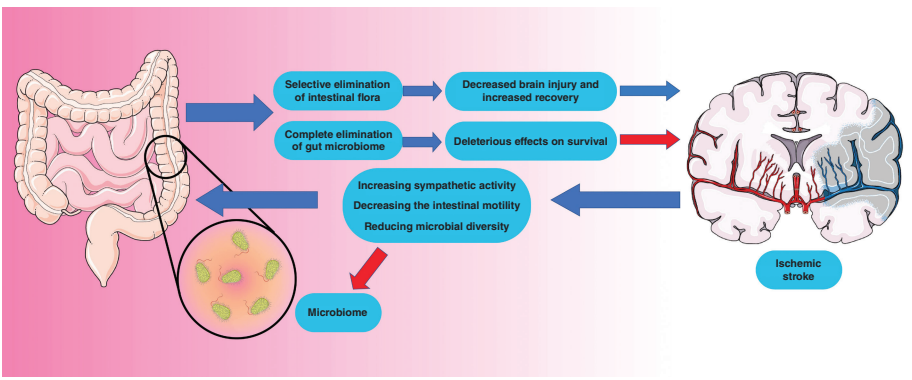

Figure 3. An overview of the gut-brain-axis after stroke. After brain injury, the gut microbiome is altered and in turn modulates stroke outcome via modulation of postischemic inflammatory responses. Stroke has been shown to affect the gut microbiome composition and diversity via increased sympathetic activity, decreasing the intestinal motility, and inducing dysbiosis. Selective removal of a certain population of intestinal bacteria has a beneficial effect on poststroke recovery. However, the complete removal of the intestinal flora has had a detrimental effect on stroke prognosis. The precise mechanisms of the gut-brain-axis after stroke are still unclear.

Neuroregenerative approaches using stem cells and EVs

The use of stem cells as a convalescent therapy after stroke has been established in animal models and is now transferred as a promising tool in human trials (94, 95). A 2019 Cochrane database analysis of randomized clinical trials of stem cell transplantation for ischemic stroke suggested an improvement in clinical outcome in patients (96). Nevertheless, the authors of the study concluded that the evidence base for an accurate assessment of stem cell therapy for the treatment of ischemic stroke is still insufficient, and further research in this field is thus urgently needed. Such research activities on both the neuroregenerative and the neuroprotective impact of stem cells in preclinical stroke models revealed paracrine mechanisms rather than cell transplantation to be the biological mediator in this respect (57). As stated before, these paracrine mechanisms gave rise to the concept of EVs. The current state of research with regard to molecular mediators transported by EVs is described in more detail below.

CHRONIC PHASE OF STROKE

The chronic phase of stroke displays distinct events such as the secondary loss of both glial cells and neurons as well as glial scar formation (97). The latter not only limits the stroke area but also blocks the synaptic connections between neurons (49). Along with this, an abnormal extracellular matrix (ECM) is formed, creating an unsuitable microenvironment for neurogenesis (98). Activation of microglia and astrocytes, as stated before, yields the production of proinflammatory cytokines that causes neuroinflammation and further deterioration of the microenvironment (99). Although BBB breakdown is a consequence of acute stroke in the first place, increased vessel leakage and risk of secondary hemorrhage can occur as a consequence of brain vasculature remodeling during the chronic stage of the disease (100). The aforementioned aspects limit neurological recovery during the chronic phase of stroke significantly. The current therapeutic approaches such as physical therapy programs and transcranial magnetic stimulation show beneficial effects for patients at the chronic phase of stroke (101, 102). However, the therapeutic effects of physiotherapy and magnetic stimulation are limited, depending on the severity of stroke lesions and the extent of neurological impairment (103). In this context, the persistence of endogenous neurogenesis within the adult mammalian brain offers new therapeutic targets during chronic stroke stages (92), albeit the majority of newborn cells would die within weeks (104). Stimulating poststroke endogenous neurogenesis by transplantion of exogenous stem cells may overcome these limitations (Figure 4).

Transplantation of stem cells

Stereotactic administration of stem cells in the proximity of the stroke area is one possible option for delivering stem cells (105). The implanted stem cells can trigger the regenerative response within the ischemic tissue, by secreting various neurotrophic factors such as ECM-modifying enzymes (106). The latter contribute to remodeling the ECM in order to improve the microenvironment for

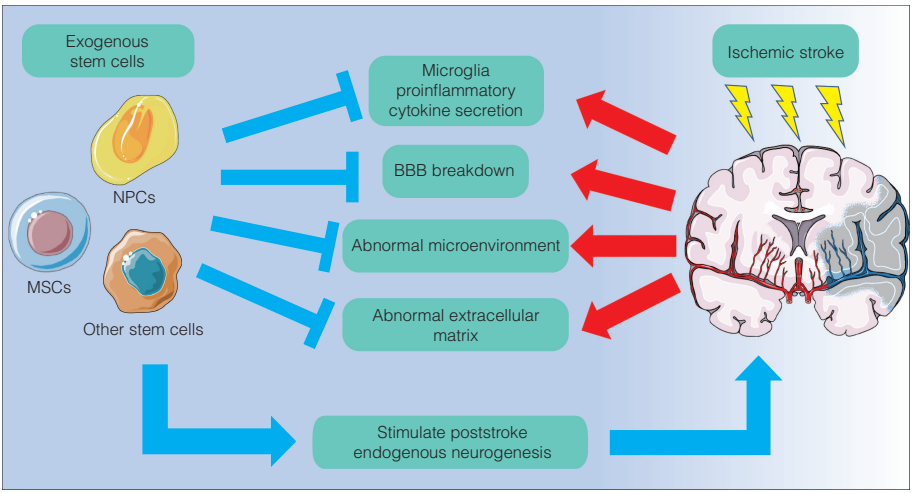

Figure 4. Transplanting exogenous stem cells supports a beneficial stroke outcome. Several pathological processes are involved in the chronic stroke phase such as blood-brain barrier (BBB) breakdown, abnormal extracellular matrix formation, and proinflammatory factors secretion from microglia, creating a hostile microenvironment that hampers neurogenesis. Exogenous stem cell transplantation can inhibit microglia activation, increase the integrity of the BBB, and form a suitable microenvironment for neurogenesis. Interestingly, the majority of exogenous stem cells do not replace damaged tissue, suggesting that exogenous stem cells act in a paracrine way. MSCs, mesenchymal stem cells; NPCs, neural progenitor cells.

synaptic regeneration. As described above, the formation of ECM limits the stroke volume but blocks the synaptic regeneration between newborn neurons. Intracerebral implantation of MSCs is considered to promote neurogenesis via remodeling the glial scar in this respect (107).

The activation of microglia and astrocytes that secrete proinflammatory cytokines such as TNF-α or IL-6 causes leukocyte mobilization in the blood and leukocyte infiltration in the brain tissue (108). These factors induce an inflammatory environment that is not conducive to neurogenesis. The reduction of inflammation levels in brain tissue due to cell therapies may also contribute to neurological recovery. Stem cells can regulate the immune response by producing antiinflammatory cytokines and thus decrease levels of activated microglia and macrophages, resulting in reduced secretion of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) (109). MSCs have been shown to reduce T-cell proliferation and activation, yielding decreased levels of proinflammatory cytokine production by monocytes/macrophages through cell-cell contact with immune cells (110, 111). Likewise, MSCs decrease the secretion of paracrine factors from microglia (such as TGF-β, indolamin-2,3-dioxygenase [IDO], and prostaglandin E2 [PGE2]), which results in the transition from a proinflammatory into an antiinflammatory state (112, 113). However, cell implantation into the periinfarct area is not a suitable method for clinical application. Other delivery methods such as systemic administration suffer from stem cell loss in peripheral organs (114, 115). However, these trapped stem cells are still beneficial in terms of enhancing poststroke neurological recovery and stimulating tissue regeneration, again demonstrating the role of paracrine mechanisms in stem cell therapy (45, 61).

Poststroke delivery of EVs

Unlike stem cells, the diameter of EVs is around 50 to 1000 nm, making EVs small enough to cross the BBB and directly affect the stroke area (56, 116). The mechanism of action of stem cell-derived EVs is mainly derived from the contents of EVs (Figure 5). The miR contents in stem cell-derived EVs have been proven to be neuroprotective or to induce neurogenesis at chronic stages of the disease (117). MSC-EV-derived miR clusters such as miR-17-92 have been proven to promote both angiogenesis and neurogenesis (118). More and more angiogenesis and neurogenesis-related miRs such as miR-133b, miR-134, and miR-181b are found in MSC-EVs in recent years (119), suggesting that miRs within EVs are involved in EV-induced angiogenesis and neurogenesis. Beside the miR content, proteins and cytokines are also involved in EV-induced neurogenesis and angiogenesis (117, 120, 121). Fibroblast growth factor 2 (FGF2), placental growth factor (PGF), and hepatocyte growth factor (HGF) that are important in adult neurogenesis are found in MSC-EVs (122). Cytokines that are related to angiogenesis, such as vascular endothelial growth factor-A (VEGF-A) and VEGF-C, are enriched in MSC-EVs as well (123). Such selected cytokines and miRs in stem cell-derived EVs support the EV-induced neurogenesis and angiogenesis after stroke (124). Beside stimulating neurogenesis and angiogenesis, EVs can also remodel the ECM (125). Evidence indicates that EVs contain ECM-related enzymes suggesting that EVs may be involved in ECM remodeling after stroke (125). MMP-9, as a key protein involved in ECM remodeling, has indeed been proven to be regulated by stem cell-derived EVs. Both MMP-9 and FGF-2 are found in mesangioblast stem cell-derived EVs (126). MMP-9 degrades gelatin within the ECM, whereas FGF-2 helps form the ECM, thus demonstrating a complex role of EVs in tissue regeneration.

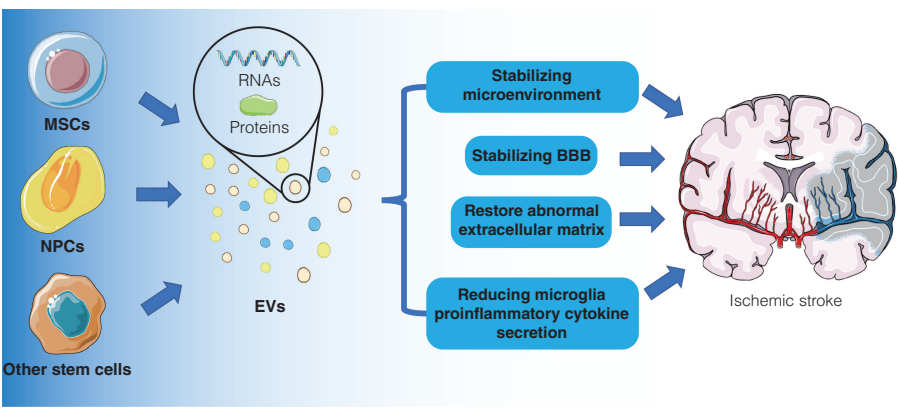

Figure 5. Stem cell-derived EVs stimulate poststroke neuroregeneration. Stem cell-derived extracellular vesicles (EVs) yield similar effects as exogenous stem cells under stroke conditions. Stem cell-derived EVs stabilize the extracellular microenvironment and enhance the integrity of the blood-brain barrier (BBB). They also reduce the proinflammatory factors secretion of microglia, contributing to a restoration of the former abnormal extracellular matrix. Abbreviations: NPCs, neural progenitor cells; MSCs, mesenchymal stem cells.

Like stem cell transplantation, stem cell-derived EVs can regulate the immune responses of the recipient (127). Stem cell-derived EVs affect the immune system after stroke mainly by regulating microglia activation in the central nervous system (CNS) and peripheral immune cells such as T cells and B cells (128). In the CNS, the microglia acts as a double-edged sword in stroke pathology. In the acute phase, activated microglia are mainly M1 type, inducing pro-inflammatiory effects (76). At this phase, M1 microglia recruit the peripheral leukocytes to the brain to clean the damaged tissues in the stroke area and cause the increase of pro-apoptotic cytokines such as TNF-α and IL-6, leading to neuron loss in the ischemic penumbra (75). However, M2 microglia are also activated in the acute phase, but their number is low. M2 microglia play an antiinflammatory role in the subacute and chronic phase of stroke (77). The M2 microglia are the majority of microglia in the CNS that release antiinflammatory cytokines such as IL-10, which redcues the overall state of inflammation in the brain but may cause immunosuppression. The latter is a common phenomenon in chronic phase stroke patients, making them vulnerable to infectious diseases. The balance between M1 and M2 microglia is one of the key points of stroke recovery (74). Stem cell-derived EVs show significant effects on regulating microglia activation (129). Several antiinflammatory miRs and proteins have been identified in human iPSC-derived neural stem cell EVs (121). Eight antiinflammatory and neurogenesis-related miRs (miRs-320a, 320b, 103a-3p, 21–5p, 26a-5p, 30a-3p, 181a-5p, 191–5p) were identified in iPSC-EVs. Among them, miR-21 has been shown to have neuroprotective and antiinflammatory properties in traumatic brain injury and stroke (130). miR-21 mediates antiinflammatory activity through the downregulation of NF-κB and TNF-α and induction of the antiinflammatory cytokine IL-10 (131). Another miR, miR-126 from ADMSC-EVs, can promote functional recovery after stroke in rats by improving neurogenesis and suppressing microglia activation (27). Meanwhile, Gal-3BP, a suppressor of inflammatory responses through NF-κB pathway, was found in iPSC-EVs. The delivery of Gal-3BP into microglia was able to restrain neuroinflammation in Alzheimer's disease (132).

The remodeling of brain vasculature with lower BBB integrity causes inefficient blood supply and increases the potential bleeding risk (100). Enhancement of neovascularization and strengthening of vascular links are therapeutic goals. Stem cell-derived EVs have shown the potential to increase BBB integrity and promote angiogenesis (41). Hence, MSC-EVs promote both angiogenesis and integrity of the BBB under chronic stroke conditions. These effects are, however, not exclusive to MSC-EVs alone. As a matter of fact, NPC-EVs also stabilize BBB integrity by decreasing NF-κB activation levels via altered ABCB1 expression patterns on the luminal endothelium (128). Along with this, EVs from endothelial cells decrease PTEN expression, stimulate AKT phosphorylation, and increase tight junction protein expression in cells, which may also contribute to enhanced BBB stability (133). Although EVs are able to enhance the integrity of the BBB under preclinical stroke settings (134), the precise mechanisms remain unclear. Further studies are therefore needed in this respect.

Stem cells and EVs under clinical stroke settings

The results of preclinical studies have encouraged the extension of stem cell transplantation and EVs to the clinic (135). Several early phase I trials have shown the

safety of MSC transplantation (136). A long-term follow-up study of autologous MSC transplantation in stroke patients shows improved recovery after stroke (137). The study included 85 patients with acute (<72 hours after onset) nonlacunar infarction within the middle cerebral artery territory who were followed up for six months. The patients were systemically administered with 5×10^7 autologous MSCs at 5 weeks and 7 weeks after stroke. SDF-1α and CXCL 12, proteins associated with MSC homing, were increased in the serum after MSC transplantation. The modified Rankin Scale (mRS) score was decreased in the MSC transplantation patients. The study proved the safety and beneficial effect of MSC transplantation. Further research showed that different dosages of MSC transplantation are safe and efficient (138). In the phase I stage, three doses (0.5, 1.0, and 1.5 million cells/kg body weight) were tested (n = 5 per cohort), and all dosages were found to be safe (138). In the phase II stage, 1.5 million cells/kg were intravenously administered to 21 patients (138). Significant functional outcome improvement was observed in 35.5% of patients at 6 months and 12 months posttransplantation. However, there are some clinical trials indicating that MSC transplantation may not affect clinical outcomes at all. A Phase I/II trial of intravenously administered autologous MSCs to 20 patients in the chronic phase of stroke between 3 months and 2 years following stroke onset did not show significant differences between the transplantation and control groups in terms of functional outcome (139). In conclusion, the completed or ongoing MSC transplantation clinical trials proved the safety of stem cell transplantation, but the effectiveness of MSC transplantation is still under debate. The data on EVs under clinical stroke settings is scarce. Only one phase I/II clinical trial currently investigates the role of allogenic MSC-derived EVs transfected with miR-124 in stroke patients (NCT03384433). The study is still ongoing, and no results are available yet.

CONCLUSION

Although recent progress has been made in modern stroke treatment, many stroke patients do not gain any benefit from it because of therapeutic limitations. Adjuvant novel treatment paradigms are therefore in order. Such experimental strategies focusing on acute therapeutic interventions primarily include neuroprotection, which has failed in clinical trials until recently. The scientific interest has now shifted towards subacute and chronic stroke stages. Transplantations of stem cells have been thoroughly investigated under such stroke conditions. However, stem cells are not integrated into residing neural networks but act via EV secretion. EV-based strategies may therefore present a next-generation therapeutic tool in stroke treatment. The advantage of such systems include the ability of EVs to cross both cell membranes and the BBB under physiological and pathological conditions alike. To the best of current knowledge, EVs have little toxic effects and no risk of tumor formation (140, 141). Serving as nanoparticle carrier, they can induce pleiotropic effects in their target organs. Nevertheless, additional studies are necessary for addressing fundamental issues with regard to technical and biological features.

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