Immunological and non-immunological effects of stem cell-derived extracellular vesicles on the ischaemic brain

Thorsten R. Doeppner, Mathias Bähr, Bernd Giebel and Dirk M. Hermann [iD]

Abstract: Following the implementation of thrombolysis and endovascular recanalization strategies, stroke therapy has profoundly changed in recent years. In spite of these advancements, a considerable proportion of stroke patients still exhibit functional impairment in the long run, increasing the need for adjuvant therapies that promote neurological recovery. Stem cell therapies have initially attracted great interest in the stroke field, since there were hopes that transplanted cells may allow for the replacement of lost cells. After the recognition that transplanted cells integrate poorly into existing neural networks and that they induce brain remodelling in a paracrine way by secreting a heterogeneous group of nanovesicles, these extracellular vesicles (EVs) have been identified as key players that mediate restorative effects of stem and progenitor cells in ischaemic brain tissue. We herein review restorative effects of EVs in stroke models and discuss immunological and non-immunological mechanisms that may underlie recovery of function.

Keywords: stroke, extracellular vesicles, neuroregeneration, post-stroke immune response

Introduction

Research activity in the field of extracellular vesicles (EVs) has significantly increased within recent years. Once thought to be cell debris or artificial *in vitro* byproducts only, EVs have now been recognized as a means of intercellular communication processes.[1,2] In spite of the significant progress made in the EV field, resulting in the formation of the *International Society for Extracellular Vesicles* in 2011, fundamental issues with regard to EV enrichment, characterization and biological properties still have to be addressed.

Yet, EVs have been recognized to not only be involved in physiological signalling cascades, but also to play a pivotal role under pathological conditions. The latter includes both pre-clinical and clinical studies on EVs as potential biomarkers for tumour formation and as therapeutic mediators against stroke and other diseases. Neuroprotection as observed after EV infusion in experimental stroke models is related to stem cell application in stroke. As a matter of fact, stem cell-induced neurological recovery after stroke is not a consequence of cell regeneration but due to paracrine mechanisms of grafted cells, among which stem cell-derived EVs are key mediators.

EV administration upon stroke is associated with a plethora of mechanisms, affecting neuroprotection, neuroregeneration and immune response. We herein discuss some of these observations made after EV application in more detail with regard to both immunological and non-immunological aspects. We conclude this review by summarizing potential obstacles and pitfalls that need to be overcome in order to establish EVs as a future adjuvant stroke treatment.

Current concepts of stroke treatment

Stroke treatment has undergone profound progress during the last decades. Once a disease that did not allow for any causal therapeutic intervention, ischaemic stroke has become a treatable disease. Current treatment concepts of stroke are based on three columns: admission of the patient to stroke units; systemic thrombolysis; and endovascular treatment.[3–7] The first stroke units, which were introduced in the 1990s, have significantly

Correspondence to:
Thorsten R. Doeppner
Department of Neurology, University Medical Center Goettingen, Robert-Koch-Str. 40, 37075 Goettingen, Germany
thorsten.doeppner@med. uni-goettingen.de

Dirk M. Hermann
Department of Neurology, University Hospital Essen, Hufelandstr. 55, 45147 Essen, Germany
dirk.hermann@uk-essen. de

Mathias Bähr
Department of Neurology, University Medical Center Goettingen, Department of Neurology, Goettingen, Germany

Bernd Giebel
Institute of Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

altered stroke therapy as it was recognized that patients treated on stroke units had better outcomes than patients not treated on stroke units.[6] In addition, the establishment of recanalizing strategies has fundamentally changed stroke treatment. Several randomized clinical trials demonstrated the efficacy of tissue plasminogen activator-induced thrombolysis that improves stroke outcome when administered intravenously within 4.5 hours after symptom onset.[8] More recently, thrombolysis has successfully been combined with endovascular recanalization therapies. Thus, patients receiving endovascular recanalization performed significantly better than patients not receiving endovascular recanalization, even when treated with intravenous thrombolytics before.[3,4] Yet, the majority of patients still present functional neurological impairment in the long run, despite optimized recanalization therapy, which raises the need for adjunct stroke treatments that allow for promotion of neurological recovery. Since the benefit of recanalization strongly depends on the time interval until recanalization is initiated, acute neuroprotection therapies may have rather limited prospects in the future, because comparable benefits can be achieved simply by modest reductions in the delay until treatment is initiated. Indeed, all neuroprotection studies using a variety of pharmacological compounds have hitherto failed in clinical trials.[9–11]

Promoting neurological recovery post-stroke by stem cell transplantation

Post-stroke brain remodelling involves a variety of different phenomena, including the de novo formation of neurons within stem cell niches like the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus.[12–16] Although endogenous neurogenesis is stimulated upon induction of cerebral ischaemia,[17,18] both survival and differentiation rates of newborn neural progenitor cells (NPCs) are limited.[19–21] In order to benefit from endogenous neurogenesis in terms of stroke therapy, however, the complex and subtle mechanisms of neurogenesis have to be understood. Indeed, Notch signalling, among others, plays a critical role in this complex process.[22] Activation of the Notch receptor induces expression of a set of various genes such as *hairy and enhancer of split 3* (Hes3) and *Sonic hedgehog* (Shh), which in turn promote pro-survival signalling cascades involving Akt, STAT3 and mTOR. All of this helps enhance the resistance of endogenous NPCs within the stem cell niche. As such, therapeutic administration of Notch ligands is associated with increased post-stroke neurogenesis, yielding better neurological recovery in stroke mice. Likewise, interfering with other signalling cascades such as the ephrin pathway offers therapeutic options as well. Indeed, knockout of ephrin B3, which is known to play a pivotal role in the formation of axonal projections, results in enhanced levels of post-stroke neurogenesis in mice.[23]

In addition to the aforementioned experimental approaches, recent efforts were made to boost post-stroke neurogenesis by transplanting adult stem cells and/or progenitor cells from various tissue sources. Indeed, the transplantation of neural progenitor cells (NPCs) and mesenchymal stem cells (MSCs) were found to enhance neurological recovery in a variety of experimental stroke models *via* mechanisms that involved the promotion of endogenous neurogenesis.[24–35] Although stem cell transplantation has profound effects on brain parenchymal remodelling, allowing for sustained neurological recovery in pre-clinical and some clinical studies,[28,30,31,33,36–43] the grafted cells exhibit poor differentiation capacity and low survival rates in the long run.[26] Together with the observation that a very low percentage of grafted cells at all reaches the brain after systemic – that is, intravenous – delivery, these findings show that the restorative effects of stem cell transplantation can hardly be explained by cell replacement, but involve indirect bystander actions. Subsequent studies suggest that soluble factors such as growth factors or cytokines secreted from grafted cells mediate stem cell actions by promoting long-term neuronal survival, neurogenesis and angiogenesis.[44] Yet, it has been shown that a large number of mediators are not released into the extracellular space by exocytosis, but loaded on EVs that are released by the cells and convey stem cell actions from cell to cell.[7,45,46]

EVs: composition and characterization

EVs form a heterogeneous group of vesicles that have recently been recognized as an additional means of intercellular signalling pathways under both physiological and pathological conditions.[1,2] The most prominent representatives of the EVs are exosomes and microvesicles. Exosomes have average sizes of 70–150 nm and correspond to the intraluminal vesicles of multivesicular bodies (MVBs) being released to the extracellular environment upon fusion of the MVBs with the plasma membrane. Microvesicles arise by the outward

budding of the plasma membrane and have an average size of 100–1000 nm. Although at the experimental level the different EV types cannot be discriminated yet, it can be considered that exosomes and microvesicles differ significantly regarding their molecular composition, which should depend on their precise origin.[1,2,47,48] The molecular content of EVs is formed by proteins, such as cell adhesion molecules, integrins, intercellular signalling molecules and membrane-organizing proteins such as the flotillins and tetraspanins, by coding and non-coding RNAs like miRNAs and by several lipids. Depending on their surface structure, EVs may selectively interact with defined target cells.[49,50] Depending on their cellular source, EVs fulfil a variety of biological functions, including pro-inflammatory and anti-inflammatory signalling.[51–56]

EVs induce beneficial effects in various disease models

To date, EVs have been used in a variety of preclinical models in order to analyse their therapeutic effects under conditions related to cerebral ischaemia and other conditions. As a matter of fact, the application of EVs derived from MSCs and other stem cell-like sources has yielded significant protection in experimental models for myocardial infarction and kidney failure, and in models of non-ischaemic neurological diseases such as amyotrophic lateral sclerosis, Parkinson's disease and myasthenia gravis.[57–72] A more detailed review of these studies is beyond the scope of this paper. Compared with these other areas, the number of studies using EVs in preclinical stroke models is small. A literature search applying the terms (in different combinations) cerebral ischaemia/stroke and extracellular vesicles/exosomes yielded a total of 301 hits as of March 2018. The majority of these papers evaluated EVs as biomarkers. Omitting these latter publications, the number of papers published in the stroke/ischaemia field using EVs as therapeutics was fewer than 40 manuscripts, including review articles.

Acute post-ischaemic neuroprotection by EVs *via* mechanisms unrelated to inflammation

In rodents, reduction of brain injury associated with neurological recovery has been reported in a number of experimental settings.[73–78] Some of these studied are summarized in Table 1. Chen and colleagues applied EVs obtained from adipose tissue derived MSCs in a rat model of focal cerebral ischaemia.[75] The authors observed a significant effect on infarct volume in the acute stroke phase. The reduction of brain injury was long-lasting, yielding increased neurological recovery that persisted over four weeks. Interestingly, additive effects were observed when MSCs and EVs were simultaneously applied in animals, suggesting different signalling pathways underlying both stem cell-based and EV-based tissue protection. On the mechanistic level, blood–brain barrier (BBB) stabilization and inhibition of apoptotic signalling were held responsible for the neuroprotective actions of EVs. Evidence for neuroprotective effects of EVs that involved anti-apoptotic actions and improved synaptic transmission were obtained in oxygen-glucose deprivation (OGD) studies *in vitro* on neurons and astrocytes.[79,80] In the immature mammalian brain – that is, in foetal or perinatal hypoxia-ischaemia – EV delivery inhibited caspase-3-dependent apoptotic cell death.[81,82]

Post-acute EV delivery induces brain remodelling and plasticity

Focusing on restorative actions in the post-acute stroke phase, Chopp and colleagues observed a significant reduction of neurological impairment that evolved over as long as four weeks after systemic delivery of MSC-derived EVs (MSC-EVs) in a model of transient middle cerebral artery occlusion (MCAO) in rats.[74] The authors attributed these effects to stimulation of post-stroke neurite remodelling as well as to stimulation of both angiogenesis and neurogenesis, all of which contribute to enhanced neurological recovery. In a head-to-head comparison, Doeppner and colleagues evaluated effects of bone marrow-derived MSCs and MSC-EVs that were administered systemically in the post-acute stroke phase, initiated 24 hours after transient MCAO in mice, demonstrating that EV delivery enhanced neurological recovery, and increased endogenous neurogenesis and angiogenesis in an almost identical way as MSCs.[73] Post-stroke brain remodelling induced by EVs involves axonal sprouting, remyelination and oligodendrogenesis, as shown by Otero-Ortega and colleagues after transient MCAO in rats,[83] and it also includes modulation of synaptic plasticity, as shown by Deng and colleagues in a model of global cerebral ischaemia in rats.[84] Notably, acute neuroprotection by EVs is not observed in these latter studies due to the delayed timing of EV delivery.[73]

The mechanisms underlying stimulation of post-stroke brain remodelling and plasticity after EV delivery are still partly elusive, although recent studies suggest an involvement of non-coding miRNAs. In rats exposed to focal cerebral ischaemia, EVs obtained from miR-133b overexpressing MSCs induced enhanced neurological recovery associated with stronger neuroplasticity when compared with EVs obtained from naïve MSCs.[77] *In vitro* experiments using OGD suggested that the enhanced efficacy of miR-133b containing EVs may be a consequence of secondary EV release from astrocytes.[77] In another study, EVs harvested from MSCs transfected with an miR-17-92 cluster plasmid induced enhanced neurological recovery when compared to EVs derived from naïve MSCs.[76] These studies suggest that miRNAs might be decisive elements mediating restorative actions of EVs. The loading of EVs with defined miRNAs is discussed to open novel ways for *ex vivo* manipulations of stem cells or EV harvests, allowing for the use of EVs as cargo carriers.[85]

Immunoregulatory effects of EVs under conditions unrelated to ischaemia

The mammalian immune system ensures host responses to infection that endanger the integrity of the whole organism. The immune system comprises the innate and adaptive immune system.[86–90] The former includes phagocytes, mast cells and natural killer (NK) cells, whereas the latter, which is also referred to as the acquired immune system, includes T and B lymphocytes. Other cells of the immune system, such as dendritic cells, form links between the innate and the adaptive immune system.

Studies on autoimmune diseases and cancer suggest a role of immune responses in mediating EV actions. Depending on their cargo (e.g. miRNAs, proteins), EVs can differentially impact immune responses.[52,91,92] Immune cells do release EVs containing immune signals, as shown for B lymphocytes, which shed EVs containing major histocompatibility (MHC) II molecules that in turn activate CD4+ T lymphocytes,[51] or as shown for dendritic cells, which secrete EVs that bind toll-like receptors (TLR), leading to spreading immune cell activation to neighbouring dendritic cells.[93] Notably, EVs obtained from synovial fibroblasts of patients suffering from rheumatoid arthritis have been shown to express higher tumour

necrosis factor (TNF) concentrations than controls not suffering from rheumatic arthritis.[94] EVs contribute to autoimmunity *via* MHC II molecules, TLR substrates and pro-inflammatory cytokines.

In contrast, EVs released from cancer cells have been shown to induce tumour angiogenesis[95–97] and suppress activity of NK cells and T cells, resulting in immune escape of tumours and tumour growth. Tumour escape is mediated by a plethora of signal cascades, including NF-kB, TNF-α, STAT-3 and other pathways.[92,98–102] The signals mediating angiogenesis still need to be characterized in further depth. Transmitting complex signals between tumour cells, immune cells and vasculature cells, EVs may give rise to the development of novel cancer therapies.

Immunoregulatory effects of EVs under conditions of ischaemia

Similar to autoimmunity and cancer, the modulation of immune signals also plays a pivotal role in responses to ischaemia. Deng and colleagues studied therapeutic effects of EVs which were obtained from human neurons, embryonic stem cells, NPCs or astrocytes on the survival of human embryonic stem cell-derived neurons that were exposed to OGD for 1 h.[79] Interestingly, all types of EVs yielded significant neuroprotection, which was associated with the inhibition of mammalian target of rapamycin (mTOR) and of various pro-inflammatory signals such as COX-2, iNOS and TNF-α as well as apoptotic signals such as caspase-3 and Bax. On the contrary, Webb and colleagues did find differences between EVs derived from different cell sources.[103] In the murine thromboembolic stroke model, the authors compared effects of neural stem cells (NSCs) with MSC-EVs, demonstrating that NSC-EVs were superior to MSC-EVs, leading to a modulation of post-stroke systemic immune response, neuroprotection and neurological recovery. The authors confirmed the efficacy of NSC-derived EVs in a model of porcine permanent focal cerebral ischaemia, where enhanced neurological recovery and improved white matter remodelling evaluated by fractional anisotropy were noted.

Although EVs are able to cross the BBB under ischaemic conditions,[104] EVs might differentially affect peripheral (i.e. in blood) and central (i.e. in brain) immune responses. In our own head-to-head

Table 1. Administration of EVs in pre-clinical stroke models and their mode of action.

In vitro/in vivo	EV source/EV isolation	Key results	Reference
In vivo (mice)	MSCs/PEG	Neurological recovery/increased angiogenesis and neurogenesis/ reversal of peripheral post-ischaemic immunosuppression	Doeppner and colleagues[73]
In vivo (rats)	MSCs/UC	Enhanced neurological recovery/ angiogenesis and neurogenesis	Xin and colleagues [74]
In vivo (rats)	Adipose derived MSCs/UC	Reduction of infarct volume/ increased neurological recovery	Chen and colleagues [75]
In vivo (rats)	Adipose derived MSCs/miRCURY	Increased functional recovery/ neuroplasticity/white matter repair	Otero-Ortega and colleagues[83]
In vivo (rats)	MSCs/UC	Enhanced neuroplasticity/increased neurological recovery	Xin and colleagues [76]
In vitro/ in vivo (rats)	miR-133b-overexpressing MSCs/UC	Secondary EV release by astrocytes/ increased neural plasticity and neurological recovery	Xin and colleagues[109]
In vivo (mice)	Embryonic stem cells/UC	Reduction of post-stroke inflammation/restoration of neurovascular unit	Kalani and colleagues[78]

EV, extracellular vesicle; MSC, mesenchymal stem cell; PEG, polyethylene glycol; UC, ultracentrifugation.

comparison, we evaluated the effects of bone marrow-derived MSC-EVs in a model of transient intraluminal MCAO and compared their effects with effects of bone marrow-derived MSC.[73] Delivery of MSC-EVs, which were administered at three time points –24 h, 72 h and 120 h after MCAO – induced sustained neuroprotection associated with neurological recovery that persisted after 28 days.[73] While no effect on the peripheral or CNS immune response was noted early after EV delivery (i.e. at 48 h post-stroke), reversal of peripheral immunosuppression was noted by flow cytometry at 144 h post-stroke. When compared to sham-operated non-ischaemic mice, vehicle-treated ischaemic mice exhibited significant B lymphocyte, T lymphocyte, monocyte and NK cell reductions in peripheral blood. The delivery of MSC-EVs reversed these changes. EV delivery did not significantly influence post-ischaemic immune cell infiltration assessed by flow cytometry at this late time point. Whether the reversal of post-ischaemic immunosuppression contributes to neurological recovery remains to be shown.

Similar observations (i.e. modulation of peripheral, but not CNS immune responses) were made by Hu and colleagues, who analysed the effects of MSC-EVs in a rat stroke model.[105] In their study, MSC-EVs did not modulate cerebral neuroinflammation as evaluated by CD45$^+$ leukocyte infiltration, but again attenuated peripheral immunosuppression seven days after stroke. Specifically, dendritic cells were elevated in the EV groups, suggesting a contribution of these cells for enhanced stroke outcome.

Evaluating the role of anti-inflammatory actions in neuroprotective effects of embryonic stem cell-derived EVs, Kalani and colleagues loaded EVs with the herbal supplement curcumin and intranasally delivered these EVs to mice exposed to focal cerebral ischaemia.[78] The delivery of curcumin-loaded EVs reduced infarct volume and decreased TNF-α levels in ischaemic brain tissue. EVs not loaded with curcumin were not examined in this study. Thus, the role of curcumin in the anti-inflammatory effects remains elusive.

To enhance cerebral accumulation of systemically delivered EVs, Tian and colleagues conjugated the cyclic derivative of the RGD peptide c(RGDyK) to the surface of curcumin-loaded

EVs followed by the intravenous infusion of these EVs in a mouse MCAO model.[106] Significantly reduced brain injury was noted in this study, which was associated with decreased cerebral inflammation, namely reduced levels of TNF- α, IL-1β and IL-6. These data again illustrate the potential of EVs as cargo carriers for anti-inflammatory molecules. Whether curcumin mediated the anti-inflammatory effects of c(RGDyK)-conjugated EVs remained again unclear.

EVs are abundantly released from stroke tissue and can be detected in peripheral blood, as suggested by Couch and colleagues, who evaluated EVs in blood samples of ischaemic stroke patients by mass spectrometry and compared the proteome patterns observed with EVs obtained from patients not suffering from a stroke.[107] In blood samples of acute stroke patients collected within the first 24 h after stroke, the authors observed significantly elevated levels of acute-phase proteins such as C-reactive protein. Interestingly, EVs derived from stroke patients were significantly increased in number. When co-incubated with cultivated macrophages, EVs obtained from stroke patients induced pro-inflammatory responses, namely increased levels of TNF- α and IL-1β.[107] Whether these pro-inflammatory actions contribute to ischaemic brain injury will have to be assessed.

Conclusion and outlook

In an evolving field, there is increasing evidence that EVs obtained from different stem and precursor cell sources potently induce post-ischaemic brain remodelling and plasticity, enabling functional neurological recovery. Thus, stem/progenitor cell-derived EVs are increasingly discussed as novel therapeutic agents in neurology. For now, neither a standardized protocol for raising stem/progenitor cells nor a standardized protocol for harvesting EVs from conditioned media exist. Recently, we compared different studies describing *in vivo* effects of MSC-EVs and realized that almost all groups use their own strategies to raise MSCs.[55] A bundle of different cell culture media has been used. Some groups use formulated media, some serum-supplemented media, and other groups, including ours, use human platelet lysate-supplemented media. Some groups change their MSC expansion media to special EV-free/reduced harvesting media; others, including us, collect conditioned media during MSC expansion and accept the presence of non-metabolized media-derived vesicles in their MSC-EV products.[55] While it may appear as an advantage to harvest MSC-EVs from specific EV-free/reduced harvesting media, it has to be considered that these media regularly do not support extensive MSC expansion that would be required to produce enough EVs for the clinical setting. Thus, in those strategies, media exchanges have to be performed from expansion to harvesting media, which will certainly affect the biology of the cells and the quality of the resulting EVs. Some groups raise their MSCs in hypoxic conditions, while others add pro-inflammatory cytokines to their MSC expansion media; both of these approaches seem to affect the EV quantity and very likely their quality as well.[55]

As different as the MSC expansion conditions are, the same holds for harvesting methods. Many groups use their own variants of differential centrifugation protocols; others use filtration-based protocols.[55] We have used a PEG-based (polyethylene glycol) purification strategy.[56] According to these variabilities, we expect that formulation and quality of the MSC-EV preparations to differ from group to group. As there are no standardized functional assays, and almost all groups use their own variation of certain functional readouts, published results can hardly be compared between the groups.[55] The issue is furthermore complicated by the fact that MSCs provide a very heterogeneous cell entity and that even upon using identical protocols for MSC expansion and EV preparation, the quality of obtained EV samples seems to differ in donor-dependent ways.[56] In the future, the ideal way to produce MSC-EV fractions for the clinical setting needs to be identified. Furthermore, despite the fact that almost all groups observe therapeutic effects of MSC-EVs in pre-clinical models, it remains an open question whether MSCs are the best EV source for the treatment of neurological diseases. Depending on the disease, EVs of other cell entities might exert different, eventually better therapeutic effects. Pre-clinical trials that compare different EV fractions side by side are mandatory to discriminate more and less effective EV production strategies for the treatment of given diseases. Furthermore, the mode of action should be identified. For now, we consider that stem/progenitor EVs execute restorative actions by mediating anti-inflammatory immune mechanisms.[55] However, in addition to these beneficial actions of anti-inflammatory stem and precursor cell-derived EVs, potentially detrimental

pro-inflammatory actions have recently been reported, which urgently need to be scrutinized. In view of the high expectations towards the clinical translation of EVs, unfavourable bystander actions need to be carefully evaluated before proof-of-concept studies in human patients may be considered. Furthermore, a number of regulatory-relevant issues have to be dealt with before EVs can be applied to humans.[108]

Conflict of interest statement
The authors declare that there is no conflict of interest.

ORCID iD
Dirk M. Hermann [iD] https://orcid.org/0000-0003-0198-3152

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