Experimental Neurology 307 (2018) 109–117

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Intravenous xenogeneic human cardiosphere-derived cell extracellular vesicles (exosomes) improves behavioral function in small-clot embolized rabbits

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ARTICLE INFO

Keywords:
Exosomes
Cytoprotection
miR
Anti-oxidant
Anti-inflammatory
Neuroplasticity
Neuroregeneration
Repair

ABSTRACT

Acute ischemic stroke is devastating to patients and their families because of few viable therapeutic options to promote recovery after reperfusion windows close. Recent breakthroughs in biotechnology have resulted in a reproducible patented process for the purification of extracellular vesicles (EVs) from human cardiosphere-derived cells (CDCs). Because CDC-EVs have many features potentially beneficial to treat acute ischemic stroke, CDC-EVs were evaluated in an established small-clot rabbit embolic stroke model, where clinically relevant end points were used to assess recovery in a more translational large animal model. Biodistribution studies with fluorescent DiD-labeled CDC-EVs showed intense uptake in the ischemic region of the brain. In this report, we show that intravenous (IV) CDC-EVs (0.75 mg/kg) administered 1-hour post-embolization significantly attenuate behavioral deficits following an embolic stroke in rabbits. In CDC-EV-treated rabbits, P_{50} (3.63 ± 1.27 mg, $n = 24$) was increased by 245% over vehicle control (1.05 ± 0.15 mg, $n = 24$); by comparison, rt-PA increased P_{50} by 91% (2.01 ± 0.24 mg, $n = 23$). Importantly, the therapy was also without adverse effects on intracerebral hemorrhage or survival rate of embolized rabbits. Thus, as a first step toward widespread use, CDC-EVs, given adjunctively to routine reperfusion therapy, merit further investigation as a therapeutic candidate for stroke

1. Introduction

Translational neuroprotection research for acute ischemic stroke (AIS) is currently undergoing a much-needed revival, in part due to the efficacy of both thrombolytic and endovascular procedures in sub-populations of ischemic stroke patients (Appireddy et al., 2016; Appireddy et al., 2015; Lapchak, 2015a). Stroke is currently treated with the Food and Drug Administration (FDA)-approved thrombolytic, tissue plasminogen activator (rt-PA), and can be treated with endovascular approaches using the stent retrievers (i.e. Solitaire and TREVO) (Powers et al., 2018), either alone or in combination with a thrombolytic (i.e.: rt-PA) prior to embolectomy for rt-PA eligible patients (Goyal et al., 2016a; Berkhemer et al., 2015; Goyal et al., 2015; Campbell et al., 2015; Jovin et al., 2015; Saver et al., 2015; Rebello et al., 2016). While rt-PA is approved and effective, it does not reduce mortality significantly, and does increase mortality due to intracerebral hemorrhage (ICH) (Whiteley et al., 2014) in certain patient populations. Moreover, even with endovascular procedures and thrombolytics, only 13.5–31% of patients undergoing the procedures end up neurologically "normal" (modified Rankin scale- mRS, 0) based upon modified Rankin scale scores, but many patients do achieve significant clinical improvement (mRS 0–2), if not a "cure" and importantly, can function independently (Goyal et al., 2016a; Klourfeld et al., 2016; Goyal et al., 2016b; Hilditch et al., 2018; Ilyas et al., 2018; Griessenauer et al., 2018).

AIS is the fifth leading cause of death and the primary cause of disability in the USA with an estimated cost of $68.9 billion annually (Lapchak and Zhang, 2017; Writing Group M et al., 2016; Benjamin et al., 2017). Each year there are approximately 800,000 new stroke victims in the USA, with 695,000 of the victims having an embolic stroke, and stroke kills 130,000 of its victims annually (Powers et al., 2018; Writing Group M et al., 2016; Benjamin et al., 2017). Ischemic brain diseases are not effectively treated due to the lack of cytoprotective therapeutics at the physician's disposal, however, reperfusion strategies such as tissue plasminogen activator (rt-PA) and embolectomy do provide effective treatment options (Henninger and Fisher, 2016). Importantly, < 15% of all patients receive or are eligible for

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https://doi.org/10.1016/j.expneurol.2018.06.007
Received 25 April 2018; Received in revised form 7 June 2018; Accepted 13 June 2018
Available online 14 June 2018
0014-4886/

treatment even with current expanded therapeutic windows. The complexity of a stroke requires novel approaches to promote clinical recovery (O'Collins et al., 2006; Lapchak and Boitano, 2017), perhaps even as an adjunct to reperfusion strategies where the adjuvant may enhance reperfusion-induced improvement.

The historical failure of poorly designed and inadequate single target neuroprotective therapy clinical trials (O'Collins et al., 2006; Lapchak and Boitano, 2017) has led to foreboding apprehension within the stroke community to even consider developing neuroprotective therapies for stroke. However, with the success of thrombectomy, a critical group of researchers and clinicians have drawn attention to the need to use adjuvant neuroprotective or cytoprotective therapies (Henninger and Fisher, 2016; Linfante and Cipolla, 2016; Lapchak, 2015b; Lyden et al., 2016). Activation of the cascade propagates a series of detrimental and devastating insults, including excitotoxicity, mitochondrial dysfunction and reduced energy metabolism, oxidative stress, inflammation, and loss of trophic support (O'Collins et al., 2006; Lapchak and Boitano, 2017; Prior et al., 2014; Pangalos et al., 2007; Swinney and Anthony, 2011). The key to success may be the use of pleiotropic cytoprotective therapies that do not specifically target one receptor or mechanism within the stroke cascade; intervention at multiple cascade targets may have significant advantages to treat stroke victims (Lapchak and Zhang, 2017; Dirnagl, 2012; Dirnagl et al., 1999; Lo and Ning, 2016; Terasaki et al., 2014; Fisher, 2011; Lok et al., 2015; Xing et al., 2012; O'Collins et al., 2011; O'Collins et al., 2012; Wahlgren and Ahmed, 2004).

Several recent studies have demonstrated that cell-derived extracellular vesicles (EVs) from various cell sources reduce ischemic burden in multiple tissues, although exosomes were originally discovered by Schubert and LaCorbiere in 1982 and called “adherons” (Schubert and Lacorbiere, 1982); these were characterized by their cellular adhesion properties with glycoproteins and glycosaminoglycans (GAGS), which may be important when targeting the stroke ischemic penumbra. Lai et al. have characterized mesenchymal EVs (Lai et al., 2016) and have shown that EVs have affinity for various lipid components, primarily monosialotetrahexosylganglioside (GM1), phosphatidylserine (PS) and globotriaosylceramide (CD77, Gb3, and ceramide trihexoside). Additionally, fibronectin on EVS may be related to the transport of EVs into brain cells (Osawa et al., 2017) after binding to heparan sulfate proteoglycan.

EVs reduce the frequency of stroke and/or ischemia-induced behavioral deficits in rodent models of stroke (Zhang and Chopp, 2016; Xin et al., 2013; Doeppner et al., 2017; Doeppner et al., 2015), and reduce infarct size and improve cardiac function in animal models of cardiac ischemia (Ibrahim and Marban, 2016; Vicencio et al., 2015; Arslan et al., 2013; Chen et al., 2013). A large repertoire of data collected to date, suggest that the functional efficacy of EVs, which include a subpopulation called exosomes, is related to their ability to modulate inflammation and attenuate oxidative stress, which reduces apoptotic cell death and potentially promotes regeneration through recruitment of endogenous progenitor cells. Exosomes are nanosized particles (~50–150 nm lipid bilayer vesicles) which can cross the blood-brain barrier (BBB) (Matsumoto et al., 2017). Exosomes and other EVs derived from progenitor cells have anti-inflammatory, anti-fibrotic and angiogenic properties, mediated by transfer of EVs payload into target cells (Marban, 2018). These characteristics make EVs a potentially important and viable therapeutic approach to treat stroke (Ibrahim and Marban, 2016). EVs from various tissue or fluid sources have different compositions, and their characteristic traits are reflected in their vesicular composition [lipid, proteins, trophic factors, small RNA]. Among the best-characterized therapeutic EV candidates are those secreted by human cardiosphere-derived cells (CDCs). These cardiac progenitor cells exert potent disease-modifying bioactivity in various ischemic models, and work indirectly via secretion of EVs [CDC-EVs] (Ibrahim and Marban, 2016; Marban, 2018; Barile et al., 2017; Gallet et al., 2017; Ibrahim et al., 2014; Nguyen et al., 2018). Among the contents which are distinctive in CDC-EVs are several miRNAs (including miR-146a, miR-181b, and miR-126) which influence therapeutic outcome after ischemia and reperfusion (de Couto et al., 2017).

In the present study, we examined whether intravenous CDC-EVs, which have potent immunomodulatory and protective properties in models of cardiac ischemia (Ibrahim and Marban, 2016; Vicencio et al., 2015; Arslan et al., 2013; Chen et al., 2013), can also reduce ischemic burden and ischemia-induced behavioral deficits in an embolic model of stroke to parallel the target patient population requiring new approaches to improve clinical function recovery. To do so, small clot-embolized rabbits were randomly allocated to receive intravenous (IV) delivery of vehicle, CDC-EVs, or rt-PA (positive control group; Genentech Inc., South San Francisco, CA) and then assessed for behavioral deficits at 48 h later, respectively. In this proof-of-concept study, we demonstrate that CDC-EVs are retained within the infarcted region of the brain, and safely and significantly attenuated behavioral deficits to a level statistically indistinguishable from that of the parallel rt-PA control ($p > .05$).

2. Materials and methods

2.1. Drug preparation & drug administration

We have isolated EVs based on previously reported methodologies (Ibrahim et al., 2014; de Couto et al., 2017). Briefly, CDCs were brought to confluence, washed 4 times with phosphate-buffered saline (PBS), and then placed in serum-free media. Fifteen days later, conditioned media was collected, filtered to remove cell debris (0.45 μm), and then concentrated using ultrafiltration by centrifugation (10 kDa molecular weight cutoff; Millipore-Sigma). All vesicles larger than 0.45 μm were removed during the first filtration step and the remaining particles < 0.45 μm were concentrated with ultrafiltration by centrifugation. The resulting particles were measured by nanoparticle tracking analysis (Nanosight®) (see Fig. 1A). The range of particle sizes (~50–250 nm and modal size of 128 nm) are shown in Fig. 1A. Quantitative PCR for microRNAs (miR)-146a, miR-181b, miR-210 is shown in Fig. 1B; miR-146a is known to be highly expressed in CDC-EVs, and transfer of miRs is one mechanism underlying the therapeutic effect of exosomes and other EVs (Ibrahim et al., 2014). As reported by (de Couto et al., 2017), miR expression was analyzed by the delta-delta cycle threshold (Ct) method (i.e.: 2–ΔΔCt method) and inert human dermal fibroblast (FB) EVs were used as controls (Ct values are provided in Table 1). The product was frozen; concentrated product was frozen until use. On the day of injection, CDC-EVs were precipitated with 5% polyethylene glycol and resuspended in saline for injection.

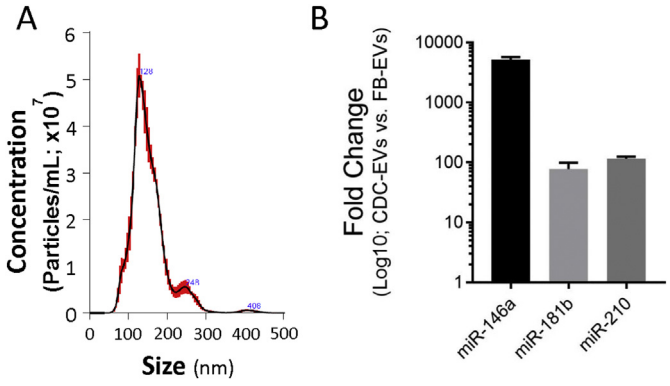

Fig. 1. CDC-EVs characterization: (A), Representative tracing of CDC-EVs concentration and size (mode = 128 nm) by nanoparticle tracking analysis. **(B),** Quantitative PCR expression analysis of miR-146a, miR-181b, and miR-210 expression in CDC-EVs relative to control fibroblast-EVs (FB-EVs). Data presented as mean ± SEM.