RESEARCH PAPER

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Hematopoietic stem cell-based angiotensin-(1-7) delivery to the brain improves functional outcome following cerebral ischemia

Aqeela Afzal[1], Fiona Desland[2], Hongwei Li[3], Robert W. Regenhardt[4], Kenneth J. Caldwell[5], Scott Barbay[6], J. Mocco[7]*

[1] Department of Neurological Surgery, Division of Surgical Sciences, Vanderbilt University Medical Center, Nashville, TN, USA
[2] Department of Internal Medicine, Mount Sinai Hospital, New York, NY, USA
[3] School of Laboratory Medicine and Biotechnology, Southern Medical University, Guangzhou, Guangdong, China
[4] Departments of Neurosurgery and Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
[5] Cancer and Blood Disorders Institute, Johns Hopkins All Children's Hospital, St. Petersburg, FL, USA
[6] Department of Physical Medicine and Rehabilitation, University of Kansas Medical Center, Kansas City, Kansas, USA
[7] Department of Neurosurgery, Mount Sinai Hospital, New York, NY, USA
**Email: j.mocco@mountsinai.org*

Angiotensin-(1-7) [Ang-(1-7)] exerts physiological effects in the brain mediated by its receptor, Mas. Recent studies have successfully demonstrated that Ang-(1-7) exerts neuroprotective effects following cerebral ischemia in a rat model. However, prior investigations utilized direct intracerebral cannulation for Ang-(1-7) delivery, potentially limiting human application. Hematopoietic stem cells (HSC) have been previously demonstrated to mobilize to the site of cerebral injury in response to stroke. Therefore, we sought to examine the therapeutic potential of HSC transduced *via* a lentivirus with Ang-(1-7) to migrate to the ischemic hemisphere and overexpress Ang-(1-7) following stroke. Animals were divided into 3 groups: Stroke + PBS, Stroke + HSC, Stroke + Ang-(1-7)-transduced HSC. Bone marrow from separate animals was harvested and used for injection of the HSC, with or without lentivirus induced Ang-(1-7) transduction. A neurological assessment was performed at 72 hours post-surgery. Ang-(1-7) transduced HSC secreted the peptide up to 72 hours post infection, *in vitro*. Stroked animals injected with the Ang-(1-7) infected HSC exhibited reduced behavioral deficits on the Bederson neurological assessment scale. These data suggest that HSC-mediated delivery of Ang-(1-7) to ischemic brain appears to improve post-stroke outcomes and may offer a novel route of therapeutic agent delivery to the brain.

Key words: Ang-(1-7), cerebral ischemia, hematopoietic stem cells

INTRODUCTION

Stroke is the leading cause of permanent disability in industrialized nations (Benjamin et al., 2018). A conceptual and technical breakthrough is imperative to identify novel targets, and strategies to cure this devastating disease. Hematopoietic stem cells (HSC) have recently been shown to mobilize to the peripheral circulation in response to stroke and to migrate to the ischemic brain (Mocco et al., 2014). The ability to genetically modify HSC may offer a breakthrough in delivering therapies previously limited by blood brain barrier impermeability. While several peptides have been delivered directly into the brain to improve neurological outcome ("A controlled trial of recombinant methionyl human BDNF in ALS: The BDNF Study Group (Phase III)" 1999; Mecca et al., 2011), direct administration has potential limitations and safety concerns. A biologic therapeutic delivery mechanism that specifically

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migrates to the site of injury may represent a promising new route for treatment delivery to the brain.

Angiotensin-(1-7) [Ang-(1-7)] is a component of the renin angiotensin system (RAS) and has been shown to exert beneficial effects in cardiovascular diseases (Ferreira et al., 2010; Xu et al., 2011; Santos et al., 2018; Paz Ocaranza et al., 2020). In the brain, Ang-(1-7) is generated predominately from angiotensin II (Ang II) by angiotensin converting enzyme 2 (ACE2), which also exists in progenitor cells (Regenhardt et al., 2014). Together, these components make up the ACE2/Ang-(1-7)/Mas axis, which counteracts a number of the deleterious effects of Ang II *via* its type 1 receptor (AT1R) in the cardiovascular system (Regenhardt et al., 2014). Our prior published data demonstrated a novel and powerful beneficial action (reduction in infarct size and behavioral deficits) of Ang-(1-7) when applied directly into the brain *via* intracerebroventricular infusion before and after ischemic stroke (Mecca et al., 2011; Regenhardt et al., 2013). Furthermore, we have demonstrated that systemic (oral) administration of a molecule where Ang-(1-7) is included within hydroxypropyl-β-cyclodextrin [HPβCD-Ang-(1-7)] after stroke affords neuroprotection, but the beneficial effects were limited (Bennion et al., 2018). The restricted beneficial actions of HPβCD-Ang-(1-7) are likely due to the fact that Ang-(1-7) has a short half-life in blood (0.5 h in humans (Petty et al., 2009) and 9 s in rats (Yamada et al., 1998) due to rapid degradation by aminopeptidases (Allred et al., 2000), and that its access to the brain is reliant upon the blood brain barrier becoming leaky after stroke. Therefore, we sought to evaluate whether using systemically applied HSC as a vehicle to deliver Ang-(1-7) to the ischemic hemisphere might elicit beneficial actions after stroke. The possibility of creating lentivirus transduced HSC to deliver Ang-(1-7) to the area of injury might indicate a viable new method to deliver neurotherapeutics. We hypothesized that the genetically modified HSC could be utilized to deliver therapeutic levels of Ang-(1-7) to the brain post stroke. By further understanding the role of HSC and the mechanisms that regulate their recruitment to the site of injury in the brain, we hope to develop therapies for stroke that can modulate HSC contribution to recovery, and potentially augment HSC's ameliorative effects, in this case with Ang-(1-7) overexpression.

METHODS

Animals

Male Sprague-Dawley rats (250-275 g) were purchased from Charles-River Farms (Wilmington, MA, USA). A total of 82 rats were used for the entire study, 48 of these were used as bone marrow donors for the HSC, 20 rats were used for the MCAO stroke model, and 14 rats were used for *in vitro* RNA analysis. This study was carried out in strict accordance with the recommendation in the guide for the care and use of Laboratory Animals of the National Institute of Health and the ARRIVE guidelines. The protocol was approved by the committee on the ethics of animal experiments of the University of Florida and Vanderbilt University. Animals were kept under specific pathogen free, well-ventilated and a temperature-controlled environment with a 12h-12h light-dark cycle and according to protocols approved under the Institutional Animal Care and Usage committee and all efforts were made to minimize suffering.

Isolation of HSC

Animals were anesthetized (n=48) with isoflurane, euthanized by decapitation and their hind legs removed. The number of enriched cells needed for each experimental group was back calculated to determine the number of donor animals needed to obtain the correct number of cells. For isolation of mononuclear cells (MNCs) from the bone marrow (BM), femur and tibia were flushed with phosphate buffered saline (PBS) +2% fetal bovine serum (FBS) and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer. Cells were spun down at 1,200 rpm for 15 min at room temperature (RT). To remove the residual red blood cells (RBCs), ammonium chloride (Stem Cell Technologies, BC, CA), was added and cells were incubated for 10 min on ice followed by washing (×2) with PBS + 2% FBS + 1 mM EDTA, to remove the residual RBCs. The resulting MNCs were re-suspended in 1 ml of PBS + 2% FBS + 1 mM EDTA and kept on ice until use.

Selection (enrichment) of CD90+/CD4.5.8- HSC from BM and blood was performed using an Immunomagnetic positive and negative selection kit, per manufacturer's protocol (Stem Cell Technologies, BC, CA). Negative selection was performed first, to exclude all CD4.5.8+ cells from the total BM MNCs. Fifty µl of Easy Sep negative selection cocktail was added per 5×10^7 cells/ml mixture in PBS + 2% FBS + 1 mM EDTA buffer, and incubated for 10 min at RT. Following this, 50 µl of Easy Sep Magnetic Nanoparticles were added to the cell mixture and incubated for 10 min at RT. Additional PBS + 2% FBS + 1 mM EDTA buffer was added to the tube to make up to 10 ml, and the tube was placed inside the Easy Sep Magnet for 10 min at RT, followed by pouring off the desired fraction into a new 12×75 mm tube. The magnetically-labeled un-

wanted cells (CD4.5.8+) remain bound on the walls of the tube, held by the magnetic field. This step is repeated one more time, and the remaining wanted cells are re-suspended in 1 ml of the PBS + 2% FBS + 1 mM EDTA buffer. Next, positive selection was performed, to select for the CD90+ cells from the remaining BM MNCs. The positive selection cocktail, including the mouse anti-CD90+ antibody, was added to the cell suspension (1:10), and incubated for 15 min at RT, followed by the Easy Sep Immunomagnetic selection as described above, three times in total. The resultant wanted magnetically labeled cells (CD90+) remained inside the tube held by the magnetic field. These cells were then resuspended in 2.5 ml of the PBS + 2% FBS + 1 mM EDTA buffer and kept on ice until use.

Transduction of HSC with lenti-Ang-(1-7)

A lentiviral construct (Fig. 1) was designed to deliver a secretable form of Ang-(1-7) from HSC. Mouse IgG2b was linked to the human prorenin prosegment which directs the translational complex to bind to the endoplasmic reticulum and produce the protein within the lumen of the endoplasmic reticulum. A furin cleavage site followed the Ang-(1-7) followed by a stop codon. The resulting transgene encodes a fusion protein capable of releasing Ang-(1-7) by taking advantage of the constitutive presence of the furin enzyme, which releases the peptide from the fusion protein along with IgG2b. The lenti-Ang-(1-7) vector has been characterized in detail in our publication (Qi et al., 2011). HSC were transduced with lenti-Ang-(1-7) at a concentration of 50 multiplicities of infection (MOI) in the presence of 8 mg/ml polybrene (Sigma-Aldrich, St Louis, Mo, USA). After 2 hours of viral transduction, the viral media was replaced with fresh growth medium. Secreted levels of murine IgG2b isotype were measured in the supernatant using a commercially available ELISA kit. The efficacy of the lentivirus in producing Ang-(1-7) was indirectly assessed by measuring secreted levels of murine IgG2b isotype in the media (Assaydesigns Plymouth, PA, USA). Supernatants were assessed at 24, 48 and 72 hours post transduction.

To further characterize the transduced HSC, mRNA from the cultured HSC at each time point (0, 24, 48 and 72 hours post transduction) was harvested and converted to cDNA using Iscript (Biorad, Hercules, CA, USA). Gene specific Taqman Assays for the Mas receptor, KDR (Kinase Insert Domain Receptor/Vascular endothelial receptor type II) and Flt1 (Fms Related Receptor Tyrosine Kinase 1/Vascular endothelial receptor type I) receptors were performed.

Induction of ischemic stroke

Implantation of intracranial guide cannula

Rats were randomly divided into three groups (Stroke + PBS, Stroke + HSC and Stroke + Ang-(1-7) transduced HSC), anesthetized with a mixture of oxygen and 4% isoflurane and placed in a stereotaxic frame. Anesthesia was maintained for the duration of surgery using a 2% isoflurane/oxygen mixture delivered to the animals through a nose cone. Body temperature and the level of anesthesia (assessed by toe pinch) were maintained throughout the surgical procedure. A 21-gauge, 4 mm long guide cannula was placed into the right hemisphere adjacent to the middle cerebral artery using the following stereotaxic co-ordinates: 1.6 mm anterior and 5.2 mm lateral to bregma, as described previously (Regenhardt et al., 2013).

Endothelin-1 induced middle cerebral artery occlusion

Three days after placement of the intracranial cannula, rats were anesthetized as described above, placed in a stereotaxic frame and underwent middle cerebral artery occlusion (MCAO) as described (Mecca et al., 2011). Briefly, an intracranial injection of ET-1 (3 µl of 80 µM solution; 1 µl/min) was delivered through the guide cannula (17.2 mm below the top of the cannula). This dosage and injection rate of ET-1 has been shown to result in ~70% reduction in regional flow from baseline cerebral blood flow (Bennion et al., 2018), body temperature and level of anesthesia were measured throughout this procedure.

Intra-arterial delivery of PBS, HSC, or Ang-(1-7) transduced HSC

Immediately after the MCAO, an incision was made in the neck region below the chin, above the rib cage. The skin was separated, and the underlying tissue dissected. The carotid artery was carefully identified, and vessel clips placed at the proximal and distal ends of the common carotid artery. All exposed tissues were kept moist using sterile saline. Rats were injected into the carotid artery with either 200 µl PBS, one million HSC re-suspended in 200 µl PBS, or with one million HSC transduced with Ang-(1-7) in 200 µl PBS while simultaneously removing the distal vessel clip. Once the injection was completed, the proximal clip was also gently removed, followed by application of Vetbond (Fisher Scientific, PA, USA) to seal the injection site. Following the injection, the common carot-

id artery was inspected for pulsatility and blood flow. All overlying tissues were placed back on top of the carotid vasculature and the skin incision closed using sterile skin staples.

Assessment of endothelin-1 induced neurological deficits

A standard neurological scale developed for rodents was used to assess a range of neurological motor deficits caused by the MCAO. Rats in each treatment group (n=8: Stroke + PBS; n=4: Stroke + HSC and n=8: Stroke + Ang-(1-7) transduced HSC) underwent neurological testing 72 hours following the ET-1 induced MCAO. Neurological evaluations were performed during daylight hours using the Bederson (1986) scale, which evaluates spontaneous activity, resistance to lateral push and circling behavior. Neurological deficits are indicated by a higher score on the Bederson scale (Desland et al., 2014).

Intracerebral infarct size

Animals were sacrificed at 72 hours post stroke by placing them under deep anesthesia with 5% isoflurane in O_2 followed by decapitation. Brains were harvested and 2 mm coronal sections were cut through the infarct zone as detailed previously (Regenhardt et al., 2013). This section was used to assess infarct volume by staining with 0.05% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 min as 37°C. Brain sections were scanned on a flatbed scanner and analyzed as described (Mecca et al., 2011), using ImageJ Software (National Institutes of Health). Corrected infarct volume was calculated to compensate for the effect of brain edema using an indirect method (Kagiyama et al., 2004). Briefly, the infarct volume was calculated using the formula: RI=LT-RN (RI: Infarct volume of right hemisphere; LT: Total volume of left hemisphere; RN: Non-infarcted cortex volume of right hemisphere).

Analysis of mRNA expression in brain tissue

A separate 2 mm coronal section was cut through the cerebrum immediately rostral to the section used for infarct volume analysis. The right cortex was dissected from this section along the line of the corpus callosum, flash frozen in liquid nitrogen and stored at –80°C until used as detailed previously (Regenhardt et al., 2013). Cortex tissue was homogenized and total mRNA isolated using the Aurum Total Fatty Fibrous kit, per manufacturers protocol (Biorad, Hercules, CA, USA). One microgram of mRNA was reverse transcribed into cDNA using Iscript (Biorad, Hercules, CA, USA). One hundred nano grams of cDNA was amplified using SsoAdvanced Universal Supermix (Biorad, Hercules, CA, USA) and gene specific (6-carboxyfluorescein labeled) Taqman Primers for the Mas receptor (catalog#: Rn00562673_m1), KDR (catalog#: Rn00564986_m1) and Flt (catalog#: Rn00570815_m1) genes (Thermo Fisher Scientific, PA, USA). An annealing temperature of 60°C followed by an extension temperature of 95°C were repeated for 40 cycles. Relative RNA levels were calculated based on Ct (Cycle time) values and normalized to a housekeeping gene (β-actin; catalog#: Rn00667869_m1).

Statistical Analysis

The individual performing the surgeries, and all subsequent analyses, was blinded as to all experimental cohorts and treatments. All statistical analysis was performed using an ANOVA with a *post hoc* Newman-Keuls Multiple Comparison test (version 5.0; GraphPad Software Inc., La Jolla, CA, USA). Data are reported as mean ± SEM, and a p value of less than 0.05 was considered to be significant and is indicated on subsequent graphs with an asterisk.

RESULTS

Transduction efficiency of the lenti-Ang-(1-7) in HSC

Transduced HSC were maintained in culture for 72 hours and the levels of secreted mouse IgG2b, which are a direct index of secreted Ang-(1-7), were measured using an ELISA (Fig. 1B). Non-transduced HSC had very low levels of IgG2b (0.3±0.3 ng/ml), which significantly increased at 24, 48 and 72 hours post transduction with the lenti-Ang-(1-7) vector (28±6, 55±4- and 85±5 ng/ml, respectively). In addition to measuring the secreted IgG2b from the transduced cells, expression of Mas, Flt1 and KDR receptor mRNAs was assessed using real time PCR (Fig. 1C). Mas and KDR expression significantly increased post transduction (3.2±0.55 and 2.1±0.26-fold changes over stroke + PBS, respectively). However, expression of Flt1 mRNA did not increase post transduction (0.07±0.07, fold change over stroke + PBS), *in vitro*.

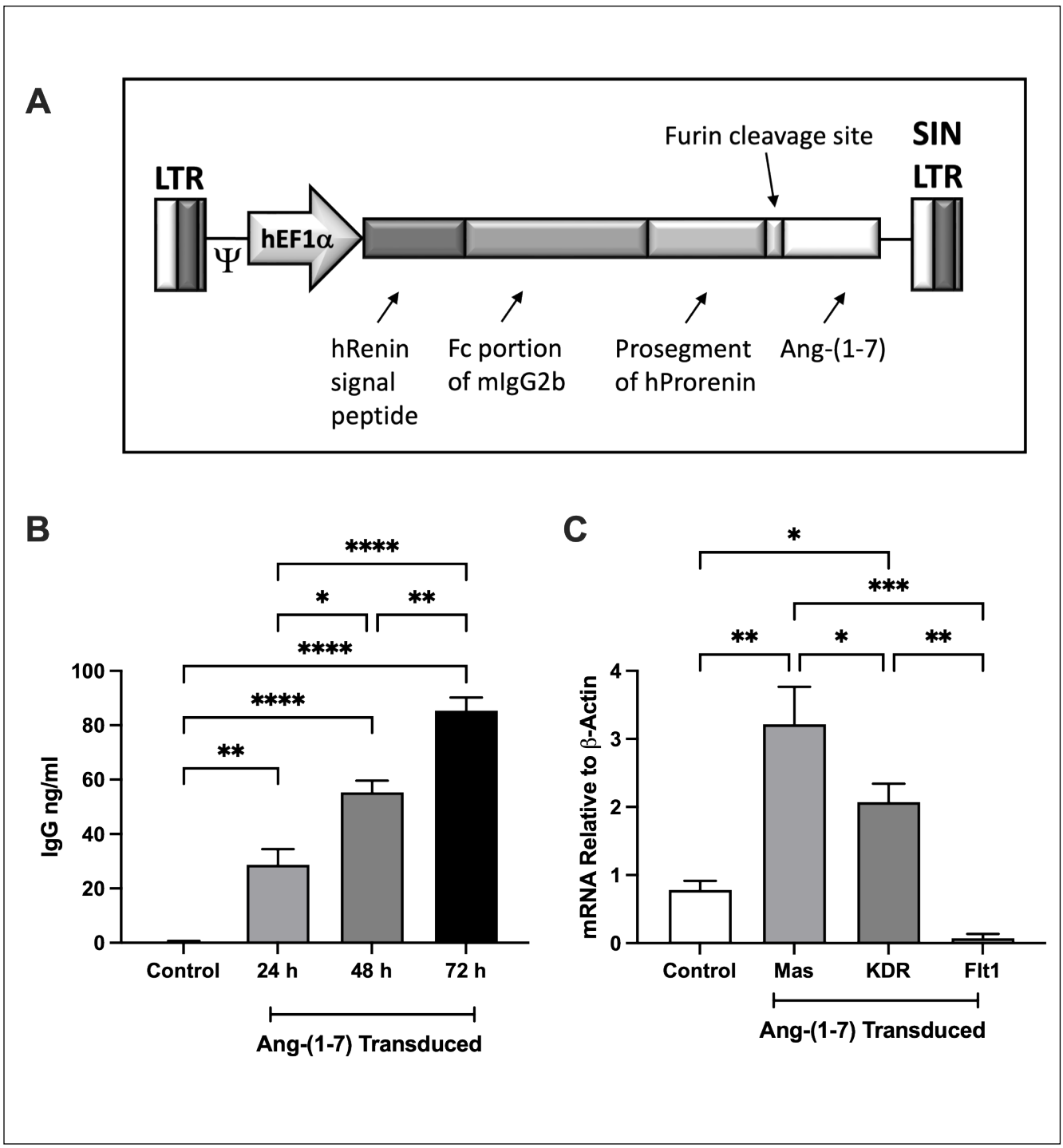

Fig. 1. Lenti-Ang-(1-7) transduction of HSC. (A) Lenti-Ang-(1-7) Construct. LTR=Long Terminal Repeat; ψ=Psi packaging signal; hEF1α=Human elongation factor 1α promoter; SIN=Self-inactivating LTR. This lentiviral construct has a mouse IgG2b upstream of the Ang-(1-7). Furin is an enzyme present in high concentrations in secretory vacuoles; the furin cleaves the mouse IgG2b away from the Ang-(1-7), thus, for every molecule of Ang-(1-7) secreted, a molecule of mouse IgG2b will also be secreted. (B) HSC were isolated from the bone marrow, infected with Lenti-Ang-(1-7) and maintained in culture for 72 hours. Supernatants from the cultured cells were analyzed for IgG levels by ELISA. Transduced cells released significantly higher amounts of IgG, which correlates to Ang-(1-7), into the media. (C) Expression of Mas, KDR and Flt1 was analyzed using gene specific primers. Transduced HSC had significantly increased expression of Mas and KDR, but not Flt1. Data are means ± SEM, n=3; *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001.

Neurological deficits following endothelin-1 induced stroke were attenuated by administration of Ang-(1-7) transduced HSC

Animals receiving ET-1 followed by intra-arterial PBS showed significant behavioral deficits using the Bederson scale (Fig. 2). These deficits were significantly less severe when Ang-(1-7) transduced HSC were administered (PBS, 1.5±0.2; HSC, 1.5±0.3; HSC + Ang-(1-7), 0.4±0.3).

Intracerebral infarct size following endothelin-1 induced stroke was not significantly altered by administration of Ang-(1-7) transduced HSC

Quantification of infarct volume using TTC (Fig. 3) demonstrated that administration of HSC alone did not alter the intracerebral infarct volume occurring after ET-1 induced stroke, when compared with control (PBS-treated) animals. In the rats administered Ang-(1-7) transduced HSC there was a tendency for intracerebral infarct volume to decrease, but the effect was not statistically significant (PBS, 48±8%; HSC, 43±8%; HSC + Ang-(1-7), 29±8%).

Ang-(1-7) transduced HSC increase the expression of mRNAs for Mas, KDR and Flt1 after ET-1 induced stroke

Brain tissue from rats that underwent ET-1 induced stroke followed by intra-arterial injection of either PBS, HSC or Ang-(1-7) transduced HSC was analyzed for levels of Mas, KDR (VEGFR1) and Flt1 (VEGFR2) mRNAs. Since Mas is the receptor for Ang-(1-7), we assessed whether an increased secretion from transduced cells had a paracrine effect on the levels of this receptor. There was a slight increase in Mas expression with HSC alone and a significantly higher expression of Mas in the Ang-(1-7) transduced HSC group (PBS, 1±0.05; HSC, 1.6±0.2; HSC + Ang-(1-7), 3.6±0.3; fold changes over PBS; $p<0.005$; $p<0.0005$). We also assessed levels of KDR and Flt1 receptors since they are well established in contributing towards angiogenesis and pro-survival, respectively (Guan et al., 2011). Expression of Flt1 mRNA was only slightly increased with HSC alone but increased significantly in the rats administered with Ang-(1-7) transduced HSC (Fig. 4B: PBS, 0.9±0.2\1; HSC, 1.1±0.07; HSC + Ang-(1-7), 2.3±0.1; fold changes over PBS; $p<0.0005$). The KDR receptor (Fig. 4C) showed a significant increase in the HSC alone and the Ang-(1-7) transduced HSC groups compared to the PBS alone group (PBS, 0.9±0.1; HSC, 1.9±0.3; HSC + Ang-(1-7), 2.7±0.2; $p<0.05$).

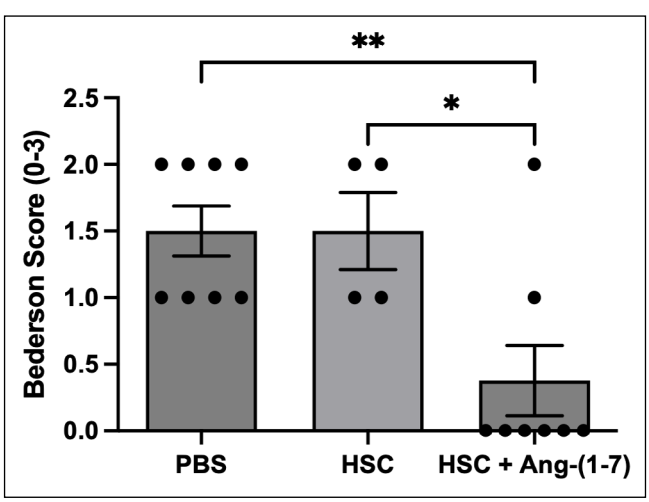

Fig. 2. Neurological effects of Ang-(1-7) treatment after ET-1 induced stroke. Rats underwent ET-1 induced MCAO followed by intra-arterial delivery of PBS (n=8), HSC (n=4) or Ang-(1-7) transduced HSC (n=8). Neurological deficits were assessed 72 hours post stroke using the Bederson neurological exam. Administration of Ang-(1-7) transduced HSC significantly reduced neurological deficits. Data are means ± SEM. *p<0.05, **p<0.005.

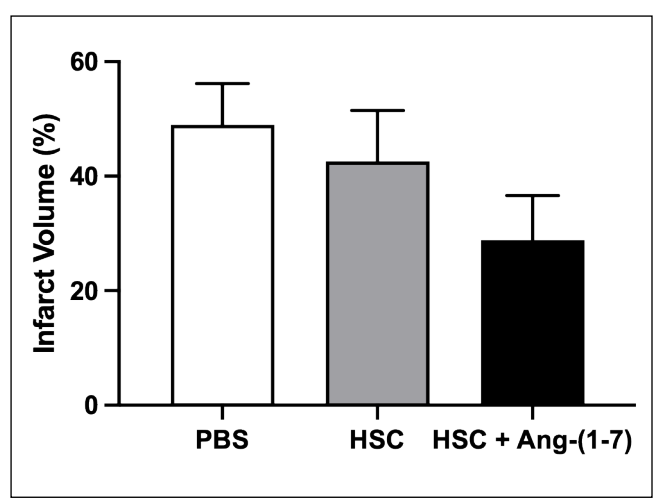

Fig. 3. Effects of Ang-(1-7) transduced HSC on infarct volume after ET-1 induced stroke. Rats underwent ET-1 induced MCAO followed by intra-arterial delivery of PBS (n=8), HSC (n=4) or Ang-(1-7) transduced HSC (n=8). Seventy-two hours post stroke, brains were removed for TTC analysis of infarct volume. Bars indicate means ± SEM showing the percentage of infarcted grey matter in each group. No significant differences were found amongst the groups.

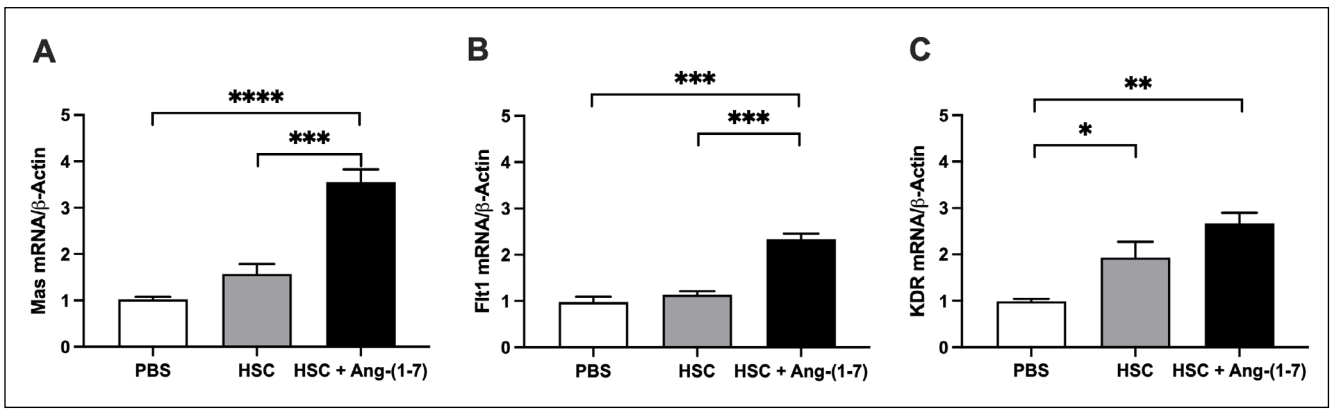

Fig. 4. Intracerebral gene expression elicited by ET-1 induced MCAO followed by intra-arterial delivery of PBS, HSC or Ang-(1-7) transduced HSC. Rats underwent ET-1 induced MCAO followed by intra-arterial delivery of PBS, HSC or Ang-(1-7) transduced HSC. Seventy-two hours post stroke, brain tissue was removed for mRNA analysis. Bar graphs shown means ± SEM of Mas (A), Flt1 (B) and KDR (C) mRNA levels from the ipsilateral hemisphere of each treatment group. *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001.

DISCUSSION

Our previous pre-clinical studies have demonstrated that either central or peripheral application of Ang-(1-7) is neuroprotective and limits cerebral damage and behavioral deficits exerted by ET-1 induced ischemic stroke (Mecca et al., 2011; Bennion et al., 2015). Importantly, there is growing evidence that axis of the renin-angiotensin system plays a role in human stroke (Bennion et al., 2016). However, there are two major translational limitations to those studies: (i) In the studies where Ang-(1-7) was delivered centrally, it was infused directly into the cerebral ventricles *via* an osmotic pump and it was administered prior to stroke induction-both of which do not hold translatable value in humans; (ii) In the study where HPβCD-Ang-(1-7) was administered orally, its neuroprotective efficacy was limited, as Ang-(1-7) entry into the brain relies upon the BBB becoming leaky (Bennion et al., 2018). Our group has previously shown that HSC cross the BBB and migrate to the area of infarct in the brain, post stroke (Mocco et al., 2014). Therefore, the goal of this study was to evaluate whether HSC could be utilized as a vehicle to deliver therapeutic levels of Ang-(1-7) to the ischemic hemisphere post stroke. Ang-(1-7) has a short half-life (seconds to minutes) (Kono et al., 1986; Yamada et al., 1998), therefore it can be challenging to maintain therapeutic levels of it in patients or animals studies. We used a gene therapy approach to mediate over expression of Ang-(1-7) by HSC transduced with a viral vector containing Ang-(1-7).

Cell transplantation into a diseased area is often injurious to the selected cell population (Robey et al., 2008). Surviving cells have to migrate, integrate and be functional to initiate repair. An intravenous (IV) delivery of HSC is the most common route for their delivery to an injured site; however, this route results in localization of a majority of these cells within the lungs, liver and spleen (Fischer et al., 2009; Harting et al., 2009; Everaert et al., 2012). To circumvent this limitation and obtain maximum delivery of cells to migrate directly to the brain, we administered the HSC intra-arterially (IA). Moniche et al. (2012) showed that an IA delivery of bone marrow mononuclear cells was safe and feasible even though no significant neurological rescue was observed. Their study is different to ours in that they administered mononuclear cells 5 to 9 days post stroke, whereas we administered transduced HSC immediately post MCAO.

A lentiviral construct has been used to deliver therapeutic levels of Ang-(1-7) for cardiovascular diseases (Qi et al., 2011). Here we used the same lentiviral vector to assess whether we could increase the concentrations of Ang-(1-7) in the brain post ischemic stroke. The lentiviral vector is designed with a furin cleavage site upstream of the Ang-(1-7). Since furin is constitutively expressed in cells, the cleavage site helps to secrete the Ang-(1-7). HSC were isolated and kept in culture for 72 hours to assess the level of mouse IgG2b in the supernatants, as a direct index of secreted Ang-(1-7). As expected, un-transduced HSC had minimal to no expression of mouse IgG2b in their supernatant and transduced cells' expression significantly increased over 72 hours post transduction. This confirmed that the HSC were indeed transduced with the lentiviral vector and the expression sustained in the HSC for at least 72 hours post transduction. Assessment of the Ang-(1-7) levels in the supernatants was indirect as the levels of mouse IgG2b were measured rather than levels of Ang-(1-7). This is an accepted and published method

of quantifying Ang-(1-7) levels (Qi et al., 2011). Furthermore, the transduced HSC had significantly higher levels of Mas receptor mRNA expression, possibly through paracrine feedback of the secreted Ang-(1-7) in the media surrounding the cells in culture. The VEGF receptors KDR and Flt1 contribute towards angiogenesis and pro-survival, respectively (Guan et al., 2011). Consistent with previously published literature there were significantly increased levels of KDR on the transduced cells, though Flt1 expression was unaltered (Sawano et al., 2001). The mRNA expression of the Mas receptor in the brain was also significantly increased in the animals that received HSC transduced with Ang-(1-7). Levels of KDR were significantly increased in the HSC alone and in the Ang-(1-7) transduced HSC brain tissues, indicating a possible shift towards a proliferative/angiogenic phenotype in the ischemic hemisphere (Eriksson & Alitalo, 2002). Levels of the Flt1 receptor in the brain tissue were not affected in the HSC alone group, but were significantly higher in the Ang-(1-7) transduced HSC group. Activation of Flt1 precedes pro-survival, stem cell recruitment and promotes vessel growth (Eriksson & Alitalo, 2002). Taken together, these data suggest that a local increase in Ang-(1-7) levels may promote new vessel growth. Our ongoing studies are focused on assessing the degree of new vessel growth in the ischemic hemisphere in response to the local Ang-(1-7) levels.

Administration of HSC alone has, in various stroke models, been shown to rescue motor deficits, with varying efficacy (Mocco et al., 2014; Afzal et al., 2024). Our results for HSC alone were equivocal, with the Bederson neurological assessment demonstrating a significant benefit for HSC + Ang-(1-7) animals. While the Bederson score is a simple and valid measure for determining deficits in acute stroker phase, it has been reported to lose sensitivity in the post stroke phase (48-72 hours post MCAO) (Bieber et al., 2019). Since our findings are based at 72 hours post MCAO, our neurological assessment might have benefitted from a modified scoring system which includes a grip test or an automated open field analysis with live video tracking of the animals (Desland et al., 2014). We acknowledge that the use of single behavior assessment is a limitation of our study and further detailed analysis is warranted for long term behavioral outcomes.

In this study we used a viral vector for overexpression of Ang-(1-7) by HSC. Further studies are needed to determine a minimal therapeutic local concertation of the Ang-(1-7) to observe similar rescue. While we have previously demonstrated that injected HSC migrate to an ischemic area in the brain (Mocco et al., 2014), we have not evaluated the efficiency with which these cells reach the infarcted region. Even though the Ang-(1-7) viral vector was designed to continuously secrete Ang-(1-7), further studies are needed to determine how many cells reach the ischemic region and contribute to the local Ang-(1-7) concentration in the brain. Additionally, our previous work has shown that the Ang-(1-7) receptor, Mas, is located on neurons, macrophages, microglia and endothelial cells of small and large cerebral blood vessels (Regenhardt et al., 2013). Stimulation of the Mas receptor with Ang-(1-7) has reportedly increased blood flow, bradykinin secretion and eNOS activity (Zhang et al., 2008). It would be interesting to further evaluate the distribution of the Mas receptor following injection of the Ang-(1-7) transduced HSC and the associated eNOS activity. Given our PCR results in this study, we would expect that the Mas receptor levels would be increased in endothelial cells lining the large cerebral blood vessels such as the middle cerebral artery (area of infarct).

TTC infarct analysis did not demonstrate a significant difference amongst the groups tested, although there was a trend for infarct size to be reduced in the HSC/Ang-(1-7) group. Despite the trend towards a reduction, no correlation between functional improvement with the infarct volume size was observed. It is possible that the functional recovery is facilitated by neuronal reorganization or rescue, rather than reduction of infarct volume (Afzal et al., 2024), although it may also be that our investigation lacked the sensitivity to detect the difference in infarct volume. Further studies will be required to determine which is the likely reason.

CONCLUSION

In summary, these data demonstrate that Ang-(1-7) transduced HSC can elicit neurorestorative molecular effects when administered post ischemic stroke and these effects may be Mas mediated. As such, HSC may be used, not only for rescue by themselves, but also facilitate delivery of neurotherapeutics to the brain. While further studies are indicated these data represent an important first step in the effort to evaluate this potential novel mechanism of stroke therapy delivery.

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