Endothelial progenitor cells as therapeutic tools in brain aging and disease

Ph.D. Dissertation

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INTRODUCTION

Societies and economies of developed countries are encountering unprecedented challenges due to the rapidly expanding older population. In 2020, the number of individuals aged over 65 reached a staggering 727 million (World Population Ageing 2020 Highlights, 2020). Projections indicate that by the year 2050, this number will more than double, with seniors constituting up to 16% of the population, equating to one in every six individuals (World Population Ageing 2020 Highlights, 2020). Aging, while not a disease in itself, brings about a series of physiological changes that are largely inevitable and correlate with a surge in brain-related diseases such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, strokes, and tumors (Azam et al., 2021).

At the tissue level, aging causes an increase in the number of senescent cells, which are incapable of proliferation and hinder the formation of new blood vessels and the repair of damaged endothelium. Endothelial senescence is linked with endothelial dysfunction, arterial stiffening, impaired angiogenesis, defective vascular repair, and an increased prevalence of atherosclerosis (Erusalimsky, 2009). At the microvascular level of the brain, aging is associated with increased capillary diameters, decreased capillary density, and heightened red blood cell velocities (Brown & Thore, 2011; Desjardins et al., 2014). Repairing brain vasculature should improve brain functions, thus we looked at ways to achieve this.

Since it was shown that reducing the number of senescent cells using senolytics can significantly increase the lifespan of mice there has been an increased interest in research studying the therapeutic use of senolytics (Xu et al., 2018). Studies have shown that senolysis in the aging brain via senolitics leads to improved blood-brain barrier (BBB) properties and reduced neuroinflammation (Yao et al., 2024). Our theory was that this treatment could be combined with endothelial progenitor cells (EPCs) therapy for a more significant effect. The positive effect of EPC therapy on neurogenesis after ischemic injury is well-documented (Esquiva et al., 2018). Still, the effect of EPCs under physiological conditions or in aging needs to be better studied.

Another field in which EPCs are promising is the delivery or in situ production of therapeutics in the brain (Cheng et al., 2023; Collet et al., 2016). As antibody therapies against misfolded proteins and their aggregates in the brain face difficulties in reaching their targets (Kouhi et al., 2021), we entered a collaboration to use EPCs as antibody factories against TDP43.

OBJECTIVES

There is no clear consensus on how endothelial progenitor cells are able to improve neural function and repair and there is little known about their function in aging. The few studies that mention the localization of EPCs study tissue regeneration in response to ischemic injury. Consequently, there is a need for more detailed research to learn whether these cells adhere to the brain vasculature transiently or are able to integrate into it. Therefore, the goals of this thesis were:

- To study the dynamics of EPC-brain vasculature interactions under physiological conditions, in aging and in response to ischemic injury.
- To study a novel approach using *ex vivo* transfected EPCs as cellular producers of antibody fragments targeting misfolded proteins implicated in neurodegeneration.
- To study whether senolytic pre-treatment using abt-263 or the combination of dasatinib and quercetin can increase EPC interactions with the brain vasculature.

MATERIALS AND METHODS

Cell culture

MAgEC10.5 and tdTomato expressing MAgEC10.5 cells (C. Kieda Patents Nr 99-16169, WO-9631178B2) were described earlier (Collet et al., 2016; Thinard et al., 2022). MAgEC10.5 cells were cultured in OPTIMEM I supplemented with 2% FBS (Thermo Fisher Scientific, Gibco, Waltham, Massachusetts USA) in a 5% CO_2 incubator at 37°C. Media was

changed every 2-3 days, depending on cell numbers. Cultures were split at or before reaching confluence. For intracarotid injections, cells were seeded at a low density (500 000 cells per 3.5 cm diameter culture dish) the day before to ensure that cells were not clumped after trypsinization. Trypsin was used at 0.05% for passage and cell collection for 3 minutes. After stopping trypsin, a small volume was used to count cells in a hemocytometer. The rest was centrifuged for 5 minutes at 4°C at 700 RCF and suspended in Ringer-HEPES so that 200 µL contained 400 000 cells.

Animals

Young, 8 to 12-weeks-old and old, 20 to 30-months-old female mice were used for aging experiments and 8-12-weeks-old mixed sex for other experiments. Mouse strains used were: BALB/c and FVB/Ant:TgCAG-yfp_sb #27. Animal housing conditions complied with the laws of Hungary (article 40/2013. (II. 14.)).

TNFα pre-treatment of MAgEC 10.5-tomato cells

MAgEC 10.5-tomato cells were seeded at 500 000 cells per 3.5 cm diameter culture dish. The following day, the cells were treated with 1 or 5 ng/mL rmTNFα (Peprotech, Margravine Road, London, UK) for 1 hour in serum-free OPTIMEM I before injection into the carotid artery of mice. To minimize the negative effects of TNFα, we selected these low doses and short treatment of the endothelial progenitor cells over systemic treatment of the mice.

Senolysis

Both young and aged BALB/c mice were administered senolytics dissolved in a vehicle mixture consisting of 60% Phosal 50 PG (Lipoid AG), 30% polyethylene glycol 400, and 10% ethanol. The first treatment protocol involved administering 100 mg/kg Abt-263 once daily for five days via oral gavage. The second protocol involved a single oral gavage of 5 mg/kg dasatinib combined with 50 mg/kg quercetin. Control groups received the vehicle mixture alone. Seven days post-treatment initiation, the mice were injected with MAgEC 10.5-tomato cells through the internal carotid artery.

Cranial window

Cranial windows were implanted similarly to previously described (Haskó et al., 2019). Anesthesia was induced with 4% isoflurane and maintained with 1.5% using an isoflurane evaporator (RWD, Guangdong, China). Using an OM-6 operating microscope (Takagi, Tokyo, Japan), the skull was gently thinned over the sensorimotor cortex in a 3-4 mm diameter circle using a micro drill (H.MH-170, Foredom, Blackstone Industries, Bethel, Connecticut, USA). The centers for the craniotomies were approximately 1.7 mm posterior and 2.2 mm lateral from the bregma. A 5 mm-diameter coverslip (Thomas Scientific, Swedesboro, NJ) was placed over the exposed brain, and Cyanoacrylate glue was used to seal the glass's edge. An aluminum head plate was cemented to the skull.

Intracarotid injection

Intracarotid injections were performed as previously described (Haskó et al., 2019). Anesthesia was induced with 4% isoflurane and maintained with 1.5% using an isoflurane evaporator (RWD, Guangdong, China). A small incision was made in the skin at the midline of the neck with a surgical scalpel. Toothed forceps were used to dissect through the muscle, exposing the carotid artery beneath. Surgical forceps were employed to separate the carotid artery from the adjacent vagus nerve. A small cotton ball, moistened with Ringer-HEPES, was placed under the carotid artery at the injection site. Loose knots were tied distally and proximally to the cotton ball, with the proximal knot tightened to prevent blood from entering the injection site. A volume of 200 µl of MAgEC cells was vortexed and drawn into a 1 ml syringe (Inject F Braun). Slowly, a 30G needle (BD Microlance 3) was inserted into the lumen of the carotid artery, positioned over the cotton ball, under the dissecting microscope.

Femoral artery cannulation and fluorescent tracer injection

Anesthesia was induced with 4% isoflurane and maintained with 1.5% using an isoflurane evaporator (RWD, Guangdong, China). The inner thigh of the mouse was shaved and disinfected with 70% ethanol and iodine. The skin was cut parallel to the femur, and connective tissue was retracted. Under an OM-6 operating microscope (Takagi, Tokyo, Japan), the femoral artery, vein, and nerve were separated bluntly. The artery was lifted from the tissue by placing three pieces of surgical thread, and three ligatures were placed. The distal ligature was tightened permanently, and the proximal was tightened temporarily. A small cut was made on the artery with micro scissors, through which the cannula was inserted and fixed in position with the middle ligation and the remaining thread of the distal one. The proximal ligature was opened to gain access to the circulation, and the skin was closed.

Two-vessel occlusion (2VO)

Briefly, anesthesia was induced with 4% isoflurane and maintained with 1.5% using an isoflurane evaporator (RWD, Guangdong, China). A 0.5-1 cm vertical skin incision was made along the ventral midline of the neck. The fat and connective tissue were lifted using forceps to access the tissue plane underneath. While observing through the microscope, the common carotid artery (CCA) was carefully separated from the surrounding nerves and veins using forceps. This procedure was repeated for both CCAs. The CCAs were gently pulled up through the silk suture and clipped with microclamps. After 30 minutes, the clips and silk sutures beneath the CCAs were removed. Finally, glue was applied to the skin wound to seal it.

Quantification of MAgEC 10.5 adhesion and immunofluorescence

Animals were transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) and fixed with 4% formaldehyde in PBS. Brains were removed and postfixed in formaldehyde at 4 °C overnight than placed in PBS and kept at 4 °C. Coronal sections were made using a vibratome (VT1000 S, Leica Biosystems, Wetzlar, Germany). PBS with 0.05% sodium azide was used to preserve 30 μm brain sections. Brain sections were temporarily mounted with PBS to count MAgEC 10.5 cells, then selected sections used for immunofluorescent labelling. Sections were incubated in PBS for 20 min at 85 °C to perform antigen retrieval. 0.5% Triton X-100 in PBS was used for permeabilisation for 1 h at room temperature. Sections were then blocked with 3% bovine serum albumin (BSA) in PBS. The primary antibodies against claudin-5 (35-2500, Life Technologies Magyarország Kft., Thermo Fisher Scientific, Invitrogen, Waltham, Massachusetts USA) were incubated overnight at 4 °C in blocking solution on a rotary shaker. Sections were washed 3 times for 5 min in PBS before secondary antibody incubation for 60 min at room temperature in the dark followed by 3 times 5 min wash in PBS. Nuclei were stained with Hoechst 33342 for 5 min, washed with PBS, and mounted with FluoroMount-G medium (Southern Biotech, USA). A fluorescence microscope (Axiovert Z1, Zeiss, Budapest, Hungary), equipped for super-resolution capable laser scanning confocal microscopy (Stedycon, Abberior Instruments, Göttingen, Germany), was used to record immunofluorescence.

Two-photon microscopy

Intravital, two-photon imaging was performed as previously described (Tóth et al., 2021). Mice were placed on a heating system after being given isoflurane anesthesia. Anesthesia was induced with 4% isoflurane and maintained with 1.5% using an isoflurane evaporator (RWD, Guangdong, China). The attached aluminum bar immobilized and held the head in place. Due to the superficial pial vasculature's consistent placement and recognizable pattern, we could capture the same cortical volume over days. Intravenous microscopy was conducted with a FEMTO 3D Dual microscope (Femtonics, Budapest, Hungary) using a 20x extended working distance water immersion objective (XLUMPLFLN-20XW, Olympus) and MES software (v6, Femtonics, Budapest, Hungary). A Mai Tai HP Ti-sapphire laser (RK TECH Ltd.) was used to optimize Venus-YFP and tdTomato. This laser was also sufficient for SR101 and optimum for EmGFP and

CellTracker Red CMTPX excitation. The laser power was varied between 10% and 40% depending on the imaging depth (0–400µm from the brain's surface). GaAsP photomultipliers were used to acquire emission wavelengths. For the first 24 hours after inoculation, larger volumes (x: 500 µm, y: 500 µm, and z: 250 µm) were recorded with three µm vertical steps to assess cell number changes and dynamics of EPCs cell integration. Image stacks were automatically merged, leveled, and RGB-converted in Fiji (software versions: ImageJ154f and Java 1.8.0_66 64bit).

***In vivo* BBB permeability measurement**

Young and old FVB/Ant: TgCAG-yfp_sb#27 mice underwent cranial window implantation for two-photon microscopy and were injected with MAgEC 10.5-tomato cells. Mice displaying red fluorescent MAgECs within the cranial window two days post-injection were then cannulated in the femoral artery and repositioned under the two-photon microscope. They were subsequently injected with Na-fluorescein and Evans blue, serving as low and high molecular weight tracers, respectively, through the cannula. Fluorescence intensity for the tracers was measured in two-photon images in regions of interest (ROI) near blood vessels containing MAgEC 10.5-tomato cells and in control vessels without them. Given the average distance between brain capillaries is approximately 40 µm, one ROI was selected immediately adjacent to the vessel, while the second was placed 20 µm away. The second ROI was chosen in a way that no other vessel was closer to it on the basis of previous three-dimensional imaging.

Quantification of MAgEC 10.5 integration

Formaldehyde-fixed 30 µm brain sections from control and 2VO mice were photographed using a fluorescence microscope (Axiovert Z1, Zeiss, Budapest, Hungary) with a digital camera and the µManager software [43, 44]. Cells were grouped based on morphology. Solid, bright cells visibly filling the vessel lumen were considered adherent. Cells showing clear endothelial morphology with thin cells surrounding the lumen with a thicker central region containing the nucleus were counted as integrated. This determination is very straightforward in vessels parallel or close to parallel with the plane of sectioning; however, the morphology of some vessels running near or at right angle with the brain section could not be determined.

2,3,5-triphenyltetrazolium chloride (TTC) staining

The brains were rapidly isolated and placed in cold PBS (0-4°C). Using a vibratome (VT1000 S, Leica Biosystems, Wetzlar, Germany), the brains were sectioned into 450 µm-thick coronal slices within 10 minutes post-decapitation. The brain slices were then immersed in TTC solution (10 ml, 2% TTC in PBS) for 10 minutes at 37°C. Following staining, the slices were fixed in 4% formaldehyde solution. Metabolically active regions stained red, whereas cerebral infarctions remained white. The sections were photographed using a Takagi OM-6 operating microscope, and infarct volumes were measured using FIJI software.

Statistical analysis

Data for each group were tested using the Shapiro-Wilk test of normality (shapiro.test()) and outliers for outliers (identify_outliers()) then ANOVA with post hoc Tukey test was performed using R and R Commander (versions 4.3.1 and 2.8-0). T-tests were calculated in Excel (Microsoft Office 2016). Data are presented in standard box plots or bar graphs of means with standard deviations, as specified in the figure legends. Statistically significant differences were indicated with their respective significance levels. All animals and measurements were included in the study.

RESULTS

MAgEC10.5 EPC adhesion to the brain vasculature

MAgEC 10.5 immortalized EPCs, labeled with CellTracker Red or red fluorescent MAgEC 10.5-tomato cells were cultured sparse (**Figure 1A**) to avoid cell clumping when injected through the internal carotid artery (ICA) of young BALB/c mice. The injected cells rapidly adhered to the vasculature and, thus most of the observed cells were in the ipsilateral hemisphere

(**Figure 1B**). In brain sections, the progenitor cells were seen inside capillary vessels and appeared to fill the lumen. However, the microvessels were likely not completely blocked, as we did not see blood cells stuck next to the progenitors in the vessel lumens after transcardial perfusion or during in vivo microscopy. Immunofluorescence labeling of neurovascular unit components, such as the collagen IV in the basal membrane allowed the monitoring of the MAgEC 10.5 endothelial progenitor cell adhesion and integration within the vessels (**Figure 1C**). Monitoring MAgEC 10.5-tomato cells through cranial windows in FVB/Ant:TgCAG-yfp_sb #27 mice that express Venus yellow fluorescent protein in endothelial cells, we observed that at this point, some of the red fluorescent MAgEC 10.5-tomato stay at the same location for 24 hours or even 5 days or longer, while others detached and moved to different sites.

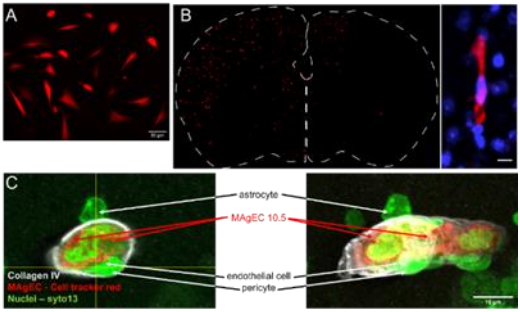

Figure 1. MAgEC 10.5 EPC adhesion in brain vessels

MAgEC10.5 EPC cell integration into the capillary bed

MAgEC 10.5 immortalized EPCs, labeled with CellTracker Red, were injected into the common carotid artery of adult wild-type BALB/c mice. As early as 28 hours post-injection, the EPCs were observed flattening along the vessel walls (**Figure 2A**), suggesting that these cells were potentially at an early step of integration into the vessel wall. For longer-term studies, MAgEC 10.5 cells were modified to express the red fluorescent protein, creating the MAgEC 10.5-tomato line, and similarly injected into BALB/c mice. Seven days after injection, the cells were analyzed for claudin 5, a tight junction protein marker. The results demonstrated the formation of tight junctions between the injected MAgEC 10.5-tomato cells and the unlabeled resident endothelial cells (**Figure 2B, C**). We concluded that the presented MAgEC 10.5-tomato cells were integrated into the capillary bed though we could only observe a partial vessel lumen as the vessel was bisected during sectioning. Later, by monitoring MAgEC 10.5-tomato cells in young BALB/c brain tissue after injection, we found that multiple cells took up endothelial-like morphology within five days. Immunofluorescence labeling and laser confocal microscopy revealed that MAgECs integrated into the capillary bed, presenting continuous tight junctions (TJs) visualized by claudin 5 staining and forming the vessel lumen together with preexisting endothelial cells (**Figure 2D**). The existence of TJs between the MAgEC 10.5-tomato derived and pre-existing cells and the presence of a vessel lumen strongly suggest that these are functional blood vessels (**Figure 2E**).

To support our ex vivo findings, we monitored MAgEC 10.5-tomato interactions with brain vasculature in vivo. MAgEC 10.5-tomato cells were injected into adult wild-type BALB/c mice, which previously underwent cranial window surgery. During this procedure, a clear glass plate was implanted into the parietal bone to allow for intravital two-photon imaging of brain tissue. 3D volume scanning with a two-photon microscope was used to monitor the integration of MAgEC 10.5-tomato cells into brain endothelial cells in vivo. Even though integration is not a

common event and the brain volume that can be monitored is restricted to an approximately 3 mm circular area and a depth of 200-400 μm, we successfully observed an integrated MAgEC 10.5-tomato cell integrated into the cortical microvasculature (**Figure 2F**). During the observation blood flow through the tdTomato positive vessel segment was clearly visible due to Cascade Blue dextran labeling.

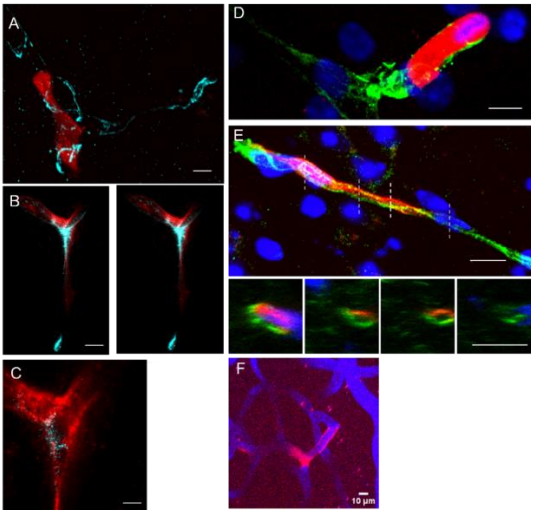

Figure 2. MAgEC 10.5 EPC integration into the capillary bed

Effect of MAgEC10.5 EPC activation, by TNFα pre-treatment, on adhesion and integration *in vivo*

Theoretically, the positive effect of EPCs on tissue regeneration can be enhanced by increasing the number of progenitors that adhere to the brain vasculature. As TNFα activates EPC adhesion in vitro (Prisco et al., 2015), we pre-treated MAgEC 10.5-tomato cells in culture for one hour with 1 or 5 ng/mL TNFα before injecting them in FVB/Ant:TgCAG-yfp_sb #27 mice. Using intravital two-photon microscopy, we saw an increased number of adherent cells 24 hours after injection. Monitoring the adherent cells for 120 hours, we found that a larger number of individual cells stayed at the same location in the vasculature compared with untreated controls.

***In vivo* BBB function at EPC adhesion sites**

To learn whether the arrest of EPCs induces changes in vascular functionality, functional assessment of the BBB and the TJs was performed by monitoring *in vivo* permeability using intravital two-photon microscopy. Young and old FVB/Ant: TgCAG-yfp_sb #27 mice underwent cranial window implantation for two-photon microscopy. The mice were injected with MAgEC 10.5-tomato. Mice in which red fluorescent MAgECs were present within the cranial window two days after injection were then cannulated in the femoral artery, placed back into the two-photon microscope and injected with Na-fluorescein and Evans blue as low and high-molecular-weight tracers through the cannula. Fluorescence intensity for the tracers was measured in two-photon images in regions of interest (ROI) near blood vessels containing MAgEC 10.5-tomato cells and control vessels that did not. The average distance between capillaries in the brain

is approximately 40 μm, thus one ROI was chosen immediately next to the vessel, while the second one was placed 20 μm farther. The second ROI was chosen in a way that no other vessel was closer to it based on previous three-dimensional imaging. ANOVA analysis showed that the BBB permeability for Na-fluorescein and Evans blue-albumin (not shown) did not significantly change either in young or old mice two days after the injection of cells. We also did not observe a gradient between the ROI closer or farther away from the vessel in question.

Detection of EPC secreted Fab in the brain

Given that MAgEC EPCs are closely associate with the brain vasculature, we tested whether these cells could be used as vectors for treating neurological disorders after genetic modification for the secretion of therapeutic molecules. Therefore, MAgEC 10.5 cells were further modified to also express the anti-TDP-43 Fab (cells named MAgEC 10.5 anti-TDP-43). Following the characterization of the cells and carotid injection into BALB/c mice, immunolabelling was used to localize the MAgEC 10.5 anti-TDP-43 cells and the anti-TDP-43 Fab expressed in relation to the brain microvasculature. The anti-TDP-43 Fab was detected in EPCs located in the brain vasculature 7 days after injection. Furthermore, anti-TDP-43 Fabs were also observed outside PECAM-1 labeled microvessels that contained red fluorescent EPCs expressing this Fab. To test whether the Fab localized into the perivascular space and penetrated into the brain parenchyma, we immunolabelled astrocytic endfeet that ensheathe the microvasculature by its specific marker aquaporin-4. We observed a distinct localization of the Fabs along the aquaporin-4 stained endfeet. From these images, it was impossible to differentiate the luminal and abluminal sides of astrocytic endfeet even using super-resolution microscopy. Nevertheless, we observed sites where the Fabs were clearly localized in the brain parenchyma, past the aquaporin-4 signal. Notably, the extravascular anti-TDP-43 Fab appeared co-located with tdTomato originating from MAgEC 10.5 anti-TDP-43 cells, suggesting vesicular localization.

Effect of hypoxia on MAgEC10.5 EPC integration and tissue regeneration *in vivo*

As tissue damage induced by ischaemic injury can be alleviated by EPC treatment, we used an experimental hypoxia model to study the role of EPC integration in this. BALB/c mice underwent two-vessel occlusion (2VO, which causes mild ischemic stroke in the anterior circulation) before being injected with MAgEC 10.5-tomato to model global hypoxia. The initial ipsilateral distribution of MAgEC 10.5-tomato cells in healthy brains shifted to a uniform distribution across both hemispheres in hypoxia. The extent of the injury was measured by comparing the area showing tissue damage to the whole area of brain sections on photographs after TTC staining. Injecting MAgEC 10.5-tomato resulted in significantly greater recovery of the ischaemic tissue. Two days after ischaemic insult, the injured brain tissue volume was 22 and 21 % for sham and MAgEC 10.5-tomato treated animals, while after 7 days it decreased to 17 and 11 %, respectively. To determine the effect of hypoxia on EPC integration, we counted MAgEC 10.5-tomato cells in tissue sections five days after 2VO and EPC cell injection. We observed a significant, approximately twofold increase in the total and integrated number of MAgEC 10.5-tomato cells in response to 2VO compared to sham-operated controls.

Effect of senolysis on the adhesion and integration of MAgEC10.5 EPCs *in vivo*

Using senolytic treatments on young and old BALB/c mice, we aimed to increase the number of EPC adhesion events by creating a niche for adhesion in place of the eliminated senescent endothelial cells. Mice underwent senolysis, then MAgEC 10.5-tomato injection, and 24 or 48 hours later, the number of adherent cells was counted (**Figure 3A**). We found that both senolytic treatments increased MAgEC10.5 adhesion to brain vasculature at both time points in young mice and after 48 hours in old mice (**Figure 3B**). The dasatinib + quercetin treatment caused a noticeably larger increase compared to abt-263. In old animals, there was a marked decrease in the number of adherent cells compared to young animals. 24 hours after injection, young animals had over 100 MAgEC 10.5-tomato cells per mm3 brain tissue while old animals had half as much.

48 hours after injection, the number of adherent cells in young animals was 63+/-4, while in old animals it was 30+/-5. Senolytic treatments were unable to completely negate the lower adhesion in old mice, even though the relative increase (compared to their respective controls) the senolytic treatments caused was similar. At 48 hours young animals had 109+/-5 or 140+/-11 adherent cells after abt-263 or dasatinib + quercetin (D+Q) pre-treatment while old ones had 42+/-8 and 53+/-8, respectively. The relative numbers of the effect of senolysis on EPC adhesion was only slightly less for old mice compared to young mice: 174%, 224% effect for abt-263 and D+Q in young animals while 140%, 176% in old animals (**Figure 3C**). As integration of the EPCs into the vasculature took five days, we tested the effect of senolytic treatment on MAgEC10.5 integration in old BALB/c mice at this time point. Even though the groups for this experiment were smaller, we found a significant increase in dasatinib + quercetin pretreated animals (**Figure 3D**). Lastly, we checked whether MAgEC 10.5-tomato cells reached other organs beside the brain after intracarotid injection, and found deficient cells counts after two days (**Figure 3E**) and none after five days (not shown).

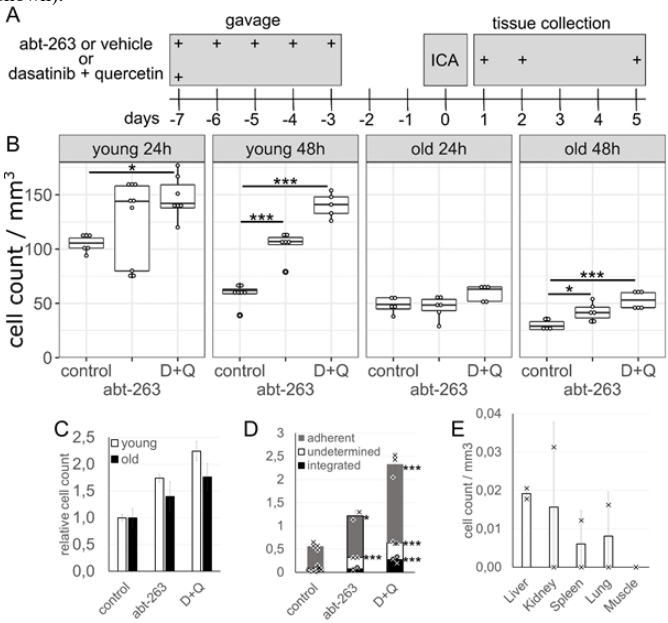

Figure 3. Effect of senolysis on EPC adhesion and integration in vivo

SUMMARY

Nowadays, aging poses a growing challenge to the society and economy of developed countries. Aging is a series of physiological processes that are generally unavoidable. However, the aging population compels society to confront the rising prevalence of ischemic stroke and neurodegenerative diseases. Aging reduces the capillary density within the brain capillary network, compromising its ability to sustain adequate CBF. Additionally, with aging, the number of senescent cells increases which induces tissue damage through increased inflammation.

Senescence is a defense mechanism against tumor development since it prevents the replication of cells with damaged genomes; however, as senescence cells accumulate, it can become pathologic.

Since 1997, the pioneering work of Asahara and colleagues has ignited interest in the study of endothelial progenitor cells (EPCs). EPCs, along with local endothelial cells in vessel walls, have the potential to repair blood vessel damage making EPCs a promising candidate for cell-based therapies. However, relatively little is known about the adhesion and integration dynamics of EPCs within the brain vasculature. This study focuses on EPC interactions with brain vasculature under physiological conditions, during aging, and after ischemic injury. A key objective was to explore a novel approach involving the *ex vivo* transfection of EPCs to serve as cellular producers of antibody fragments targeting misfolded proteins associated with neurodegenerative diseases. Another key goal was to improve the efficacy of EPC therapy. To this end we eliminated senescent cells using senolytics to potentially make room for more EPC integration. Another avenue we explored was to increase the cell adhesive properties of EPCs by activating them before injection.

We utilized an immortalized cell line derived from embryonic mouse aorta–gonad–mesonephros endothelial cells (MAgEC 10.5). To enable *in vivo* tracking during long-term experiments, MAgEC 10.5 cells were transduced to express the red fluorescent protein tdTomato, this daughter cell line was named MAgEC 10.5-tomato. Additionally, MAgEC 10.5 cells were modified to express anti-TDP-43 Fabs to investigate their potential as a vector for local expression of therapeutic molecules in the diseased tissue. We employed intravital two-photon microscopy to monitor fluorescently labeled MAgEC 10.5 *in vivo* and immunohistochemistry combined with laser scanning microscopy to analyze the behavior of these cells *ex vivo*.

Fluorescent MAgEC 10,5 EPCs were injected into the common carotid artery of BALB/c mice. After injections, the brains were sectioned at various time points. We observed that most MAgEC 10.5 completely filled the capillary lumen in brain sections after injection. Later, some EPCs were flattened against and stuck to vessel walls. After about five days we observed a few MAgEC 10.5 cells integrated into brain capillaries. We were the first to show that EPCs adherent to the brain vasculature integrated into microvessels and took up endothelial morphology, established tight junctions, and formed vessel lumens together with neighboring endothelial cells. Injected MAgEC 10.5 were almost exclusively detected in the hemisphere ipsilateral to the injection, suggesting that EPCs adhered to the vasculature shortly after injection and did not pass through the circulation multiple times. Early adhesion of EPCs in brain vessels after intracarotid injection was further supported by our finding of meager numbers of MAgEC 10.5 cells in peripheral tissues.

To assess the dynamics of EPC-brain vasculature interactions for longer studies, we followed MAgEC 10.5-tomato cells in the brain of FVB/Ant: TgCAG-yfp sb #27 mice that express the Venus yellow fluorescent protein in endothelial cells by intravital two-photon microscopy. It was rare for MAgEC 10.5-tomato cells to stay at the same location for multiple days unless the cells were activated previously by TNFα. TNFα not only increased the number of adherent cells but also prolonged their adhesion at the same location. We also showed that the arrest of EPCs induced no changes in blood-brain barrier function, based on *in vivo* permeability assays using intravital two-photon microscopy with classical fluorescent tracers.

Since MAgEC 10.5 cells can remain attached to brain vasculature for several days, they may serve as a means for local delivery of therapeutic agents. Our collaborators created MAgEC 10.5 derivative cell lines that release therapeutic fragment antigen-binding regions or Fabs. They showed that the Fabs secreted by these cells can solubilize the neurotoxic β-amyloid or TDP-43 protein aggregates *in vitro*. To evaluate whether this system can be used to introduce the therapeutic Fabs into the central nervous system. Our results showed that MAgEC 10.5 cells expressing anti-TDP-43 Fabs retained the transgene expression after adhering to the brain

vasculature *in vivo*. Most of the secreted Fab is localized at the vessel wall, but some could be detected in the brain parenchyma as well. These results demonstrate that antibodies secreted at the brain vasculature by transgenic EPCs can successfully penetrate into the brain parenchyma. The use of MAgEC 10.5 cells expressing anti-TDP-43 Fabs has two key aspects. First, the EPCs themselves help repair the damaged vasculature. Second, the cells secreted the therapeutic Fab at the site of therapy and introduced it into the brain parenchyma where it could help treat ALS by solubilizing TDP-43 aggregates.

We investigated the integration of MAgEC 10.5-tomato cells using the 2VO model, employing the MAgEC 10.5-tomato cell line to show that it acts similar to the cell types used by others - in that it alleviates detrimental effects after ischemic injury and to study whether the integration of these cells could play a role in this. Our findings revealed significant tissue regeneration in mice injected with MAgEC 10.5-tomato cells following ischemic damage. Concurrently, the number of MAgEC 10.5-tomato cells in brain tissue and, more importantly, the number of integrated MAgEC 10.5-tomato cells, was significantly increased.

Since senolysis eliminates senescent cells from tissues, we hypothesized that this process could enhance the adhesion of circulating EPCs by making space with the removal of the senescent cells. To test this hypothesis, we injected red fluorescent MAgEC 10.5-tomato cells into mice pretreated with senolytics. We found that the two senolytic treatments we used, Abt-263 and the dasatinib-quercetin combination both significantly increased the interaction of MAgEC 10.5-tomato cells with the vasculature in both young and old mice. Furthermore, we demonstrated that both senolytic treatments enhanced the integration of MAgEC 10.5-tomato cells in old mice.

In conclusion, EPCs rapidly adhere to the vasculature following injection. We demonstrated that EPCs integrate into microvessels and contribute to vessel formation, underscoring their efficacy in promoting brain tissue healing after injury. Pre-activation of EPCs enhanced their interaction with the vasculature. TNFα emerged as a promising candidate for this purpose. Genetically modified EPCs can be used as a platform to introduce therapeutic Fabs directly into the brain parenchyma. EPCs adhered less to old brain vasculature compared to young brains, which was counteracted by combining EPC injections with senolytic therapy. Furthermore, we demonstrated not only increased adhesion but also increased integration of EPCs in response to ischemic injury that may form the basis of the regenerative potential of EPC-based treatment. Thus our study highlights the therapeutic potential of injected EPCs in the brain vasculature which can be further enhanced using senolytics, EPC activation, and genetic modification of EPCs.

ÖSSZEFOGLALÁS

Az agy működéséhez nélkülözhetetlen a folyamatos és egyenletes tápanyagellátás valamint a felesleges anyagcseretermékek eltávolítása. Ezt az agyi véráramlás valamint az érfalon keresztüli anyagtranszport pontos szabályozása biztosítja. Ez utóbbiért a vér-agy gát alkotásában kulcsfontosságú agyi endotélsejtek felelnek. A vér-agy gát sok gyógyszerhatóanyag agyba jutását is megakadályozza, ezáltal nehezítve hatékony terápiák kialakítását. Agyi trauma, neurodegeneratív és neurovaszkuláris megbetegedések valamint öregedés során az agyi ereket belülről burkoló endotélsejt réteg sérül. A sérülések kijavításában fontos szerepet játszanak az endoteliális progenitor sejtek (EPC), melyek száma megemelkedik az agyi ereket érintő több betegségben. Az öregedés során nő a szeneszcens sejtek száma. Az osztódásra képtelen, apoptózisnak ellenálló szeneszcens sejtek hozzájárulhatnak a szöveti károsodáshoz az általuk kiválasztott gyulladásos faktorok által. A közelmúlt felfedezése, hogy a szeneszcens sejtek száma szenolitikumokkal csökkenthető, ami egerekben az élettartalm megnövelésével is járt. Kísérleteink során vörös fluoreszcens fehérjét kifejező EPC sejteket injektáltunk egerek belső nyaki verőerébe majd ezek sorsát követtük agyszeletekben epifluoreszcens és lézer-konfokális mikroszkópiával illetve élő egér agyban intravitális két-foton mikroszkópiával. Az agyi keringésbe jutó EPC-k rövid idővel az injekció után az agyi erek felszínéhez tapadtak és nem

jutottak el jelentős számban más szövetekbe. Ugyanakkor az agyban viszonylag rövid ideig voltak megfigyelhetők egy-egy pozícióban, gyakran helyet változtattak. Mivel az EPC-k jelentős számban elsősorban az agyban maradtak több napon keresztül, ígéretes hordozók lehetnek agyi megbetegedések elleni gyógyhatású anyagok számára. Ennek tesztelésére, módosított EPC-t használtunk, amely az ALS-re jellemző patológiás TDP-43 fehérjeaggregátumok felbontására alkalmas antitestet termel. Az egerek keringésébe adott módosított EPCk antitest termelése működőképes volt miután a sejtek eljutottak az agyba és a termelt antitest kimutatható volt az agyszövetben. Tehát módosított EPC-kkel helyben termeltethetünk terápiás molekulákat a sérült agyszövetben. Az agyi kapillárisok falába kisszámú EPC épült be egészséges agyban, viszont, iszkémiás sérülést követően nőtt az erek falához kitapadt sejtek száma és több is integrálódott az érfalba. Egy az iszkémiát követően megemelkedett szintet mutató gyulladásos faktor, a TNFα segítségével iszkémia nélkül is növelni tudtuk az érfalhoz tapadó EPC-k számát. Ez a kezelés növelte azt az időt is, amit egyes EPC-k egy helyben töltöttek. Öreg egerekben a várakozásunkkal ellentétesen kisebb számú EPC tapadt ki, mint fiatal állatokban. Ugyanakkor, bizonyos mértékben növelni tudtuk az EPC kezelés hatékonyságát szenolitikus kezelésekkel. Ezek alapján állíthatjuk, hogy az EPC alapú kezelés alkalmas lehet az öregedés és az öregedéssel kapcsolatos megbetegedések hatására bekövetkező érsérülések kezelésére, különösen, ha együttesen alkalmazzuk a hatékonyságát növelő, pl. szenoterápiás kezeléssel.

LIST OF PUBLICATIONS

MTMT Number: 10090447

Mandatory peer-reviewed international publications for the fulfillment of the doctoral process and on which this thesis is based:

1. **Lam Tri Duc**, István Tóth, Anca Hermenean, Imola Wilhelm, Claudine Kieda, István Krizbai, Attila E. Farkas (2024). **Senolysis potentiates endothelial progenitor cell adhesion to and integration into brain vasculature**. https://doi.org/10.1186/s13287-024-04042-2 . Stem cell research and Therapy 2024. **Impact Factor: 7.1 (D1)**
2. Thinard, R., Farkas, A. E., Halasa, M., Chevalier, M., Brodaczewska, K., Majewska, A., Zdanowski, R., Paprocka, M., Rossowska, J., **Duc, L. T**., et al (2022). "**Endothelial antibody factory" at the blood brain barrier: Novel approach to therapy of neurodegenerative diseases**. *Pharmaceutics*, 14(7), 1418. DOI: 10.3390/pharmaceutics14071418. **Impact factor: 5.4 (Q1)**

Total impact factor of publications used in the dissertation 12,5.

The other publications:

3. Lee, B., Lee, M., Song, S., Loi, L. D., **Lam, D. T**., Yoon, J., Baek, K., Curtis, D. J., & Jeong, Y. (2017). **Specification of neurotransmitter identity by Tal1 in thalamic nuclei**. Developmental Dynamics, 246(10), 749–758. https://doi.org/10.1002/dvdy.24546. **Impact factor: 2.507 (Q2)**

4. Lee, M., Yoon, J., Song, H., Lee, B., **Lam, D. T**., Yoon, J., Baek, K., Clevers, H., & Jeong, Y. (2017). **Tcf7l2 plays crucial roles in forebrain development through regulation of thalamic and habenular neuron identity and connectivity**. Developmental Biology, 424(1), 62–76. https://doi.org/10.1016/j.ydbio.2017.02.010. **Impact factor: 3.262 (Q1)**
5. Lee, B., Yoon, J., **Tri Lam, D**., Yoon, J., Baek, K., & Jeong, Y. (2017). **Identification of a conserved cis -regulatory element controlling mid-diencephalic expression of mouse six3**. Genesis, 55(3), e23017. https://doi.org/10.1002/dvg.23017. **Impact factor: 2.667 (Q1)**
6. Lee, B., **Tri Lam, D**., Baek, K., Yoon, J., & Jeong, Y. (2015). **Conditional cell ablation via diphtheria toxin reveals distinct requirements for the basal plate in the regional identity of diencephalic subpopulations**. Genesis, 53(6), 356–365. https://doi.org/10.1002/dvg.22857. **Impact factor: 2.667 (Q2)**

Total impact factor of all publications: 23.6

Conferences:

Lam Tri Duc, István Tóth, Anca Hermenean, Imola Wilhelm, Claudine Kieda, István Krizbai, Attila E. Farkas. **Brain vasculature rejuvenation by combined senolytic and progenitor cell therapy**. 26th International Symposium on "Signal Transduction at the Blood-Brain Barriers, Szeged, Hungary, September 11-13, 2024

Lam Tri Duc , István Tóth, Imola Wilhelm, Claudine Kieda, István Krizbai and Attila Farkas. **Hypoxia and inflammation induce adhesion and incorporation of Endothelial Progenitor Cells in the brain vasculature**. Straub Napok, HUN-REN Biological Research Centre, Szeged, Hungary, May 30-31

Attila Farkas, Lam Tri Duc , István Tóth, Imola Wilhelm, Claudine Kieda, István Krizbai. **Intergration of endothelial precursor cells into the brain vasculature. 24th International Symposium on Signal Transduction at the Blood-Brain Barriers**. Bari, Italy, September, 21-23, 2022

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DECLARATION

I declare that I know the contents of the dissertation entitled "**Endothelial progenitor cells as therapeutic tools in brain aging and disease**" written by the PhD candidate **Lam Tri Duc**. As corresponding author of the first article and co-first author of the second article and as supervisor of Lam Tri Duc, I declare that the contribution of Lam Tri Duc was significant in these publications and that the PhD dissertation is based on data presented in these publications. I confirm that the results reported in the PhD dissertation were not used to acquire any other PhD degree in the past and will not be used in the future either.

1. **Lam Tri Duc**, István Tóth, Anca Hermenean, Imola Wilhelm, Claudine Kieda, István Krizbai, Attila E. Farkas (2024). **Senolysis potentiates endothelial progenitor cell adhesion to and integration into brain vasculature**. Stem cell research and Therapy 2024. https://doi.org/10.1186/s13287-024-04042-2 . Impact Factor: 7.1 (2023)
2. Thinard, R.; Farkas, A.E.; Halasa, M.; Chevalier, M.; Brodaczewska, K.; Majewska, A.; Zdanowski, R.; Paprocka, M.; Rossowska, J.; **Duc, L.T**.; et al. **"Endothelial Antibody Factory" at the Blood Brain Barrier: Novel Approach to Therapy of Neurodegenerative Diseases.** Pharmaceutics 2022, 14, 1418. 10.3390/pharmaceutics14071418. Impact Factor: 5.4 (2022)

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