Research report

Endothelial progenitor cell transplantation restores vascular injury in mice after whole-brain irradiation

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HIGHLIGHTS

• Levels of endothelial progenitor cells (EPCs) are decreased in the peripheral blood following irradiation exposure.
• EPC transplantation may help to restore damage to the blood–brain barrier, tight junctions following irradiation exposure.
• EPC transplantation may increase cerebral capillary density after irradiation exposure.

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ABSTRACT

Vascular damage plays an important role in the pathogenesis of radiation-induced brain injury (RBI). Endothelial progenitor cells (EPCs) are responsible for maintaining and repairing endothelial function, and have become a promising method for the treatment of cerebrovascular diseases. However, whether EPC transplantation plays a protective role in RBI has not been fully elucidated. Therefore, the present study investigated the effects of bone marrow-derived EPC transplantation in a whole-brain irradiation (WBI) mouse model. Mice were divided into the three groups: control group, irradiation group and EPCs group. Phosphate buffered saline or EPCs were intravenously injected into mice one week after irradiation, and brains were analyzed eight weeks after injection. Flow cytometry demonstrated that irradiation led to a significant reduction in the peripheral blood EPC count; however, EPC transplantation led to a significant increase in the circulating EPCs. Intravital two-photon imaging and western blotting demonstrated that EPC transplantation reversed the effects of irradiation by decreasing blood–brain barrier permeability and increasing the expression of tight junction proteins in the brain. Additionally, immunofluorescence staining revealed that the brain microvascular density was higher in the EPCs group than the irradiation group. Therefore, EPC transplantation may restore damage caused by WBI to the blood–brain barrier, tight junctions, and cerebral capillary density. These results highlight the potential beneficial effects of EPC transplantation on vascular damage induced by RBI.

1. Introduction

Radiation therapy is widely used for the treatment of diseases such as primary head and neck tumors and cerebral metastatic tumors (Makale et al, 2017; Guckenberger et al, 2018). Radiation therapy can improve the survival rate of patients. However, it also affects the healthy surrounding tissue. Radiation-induced brain injury (RBI) is a serious complication of cranio-cerebral radiotherapy, and can cause injuries to the brain tissue, such as necrosis, edema, demyelination, and impairment of cognition and memory (Helson, 2018; Greene-Schloesser and Robbins, 2012). Many studies have described the pathophysiologic mechanisms of radiation-induced brain injury, including vascular injury, inflammatory immune response, and parenchymal damage, among others (Balentova and Adamkov, 2015). Vascular injury is considered to play a critical role in RBI.

Since endothelial progenitor cells (EPCs) were identified in the human blood (Asahara et al, 1997), they have attracted attention because of their potential for therapeutic and prognostic applications.
EPCs, derived from bone marrow or umbilical cord blood, are precursor cells of endothelial cells. EPCs are able to mobilize to damaged vessels, proliferate, and differentiate into mature endothelial cells (Wils et al., 2017). EPCs play an important part in angiogenesis, and may contribute to the restoration of the blood–brain barrier (BBB) damage.

EPC therapy is a promising treatment strategy for both cardiovascular and cerebral ischemic diseases (Fan et al, 2010; Leistner et al., 2011). To the best of our knowledge, only a few studies have assessed the relationship between RBI and EPCs. Thus, it is not clear whether EPCs have a protective role with regard to RBI. Researchers have shown that EPC levels are markedly reduced in the bone marrow and peripheral blood after irradiation exposure (Ashpole et al., 2014). Further, vascular networks are severely damaged after radiation. Given the angiogenic property of EPCs, we hypothesized that cerebral vessel recovery failure after whole-brain irradiation might be due to a decline in EPCs, and that EPC transplantation may have protective effects against RBI. In the present study, we investigated the utility of EPC transplantation for the treatment of RBI, as well as the possible underlying mechanisms (Fig. 1).

2. Results

2.1. Characterisation of EPCs

After culturing mononuclear cells (MNCs) from the bone marrow of a mouse in endothelial basal medium-2 (EBM-2), putative EPCs attached to the plate and exhibited a “spindle” or “cobblestone” appearance under a light field microscope (Fig. 2A). Immunostaining indicated that approximately 95% of the adherent cells expressed the surface markers of EPCs (CD34, VEGFR2, CD133, and CD31) (Fig. 2B). EPCs were labeled with DiI to track the transplanted EPCs in mice. DiI-EPCs were detected in the brain tissue by fluorescence microscopy one week after transplantation (Fig. 2C). No labeled cells were observed in the control and irradiation (IR) groups.

2.2. Effect of WBI on circulating EPC counts

Blood-derived mouse EPCs were isolated 8 weeks after WBI and were quantified by flow cytometry. EPCs that were positively stained for both CD34 and VEGFR2 were quantified. As shown in Fig. 3, blank and single-stained cells were used as controls. The percentage of EPCs in peripheral MNCs was (0.42 ± 0.06) % in the control group and (0.17 ± 0.02) % in the IR group. The EPC count in the peripheral blood was significantly reduced after WBI compared to the control group (p < 0.001). After cell transplantation, the number of circulating EPCs in the transplantation group (0.34 ± 0.03) % was significantly increased compared with the IR group (p < 0.01).

2.3. EPC transplantation reduced leakage of Rhodamine B after WBI

To investigate the permeability of the BBB, Rhodamine B was intravenously injected to detect dye leakage. As shown in Fig. 4B, Rhodamine B leakage was observed from the vessels into the extravascular compartment in the IR and EPCs groups, but not in the control group. The average fluorescence intensities in the extravascular space amongst the groups at different timepoints were compared using a two-way repeated measures ANOVA. There was a significant interaction between time and treatment (F = 7.840, p < 0.05). Therefore, a simple main effects test was performed as a post hoc analysis. Our results revealed that 5 min after injection of the dye, none of the groups showed significantly different extravascular fluorescence intensity. At 15, 30, 45, and 60 min after the injection, the intensity of the IR group was significantly higher than the control group. There was no significant difference between the IR and EPCs groups at 15 min; however, the intensity in the EPCs group was significantly lower than the IR group at 30, 45, and 60 min.

2.4. EPC transplantation increased the expression of claudin-5 and zonula occludens-1

To measure the expression of tight junctions in the mouse brain, western blotting and immunofluorescence staining were performed to detect claudin-5 (CLDN5) and zonula occludens-1 (ZO-1), which are specific markers for tight junction proteins. The expression of claudin-5 and ZO-1 were significantly reduced after irradiation compared with the control and EPCs groups, as shown by western blot (Fig. 5). Similar results were observed with immunofluorescence staining (Fig. 6). This shows that WBI can lead to injuries in the BBB, and that EPC transplantation may help to restore this damage.

2.5. EPC transplantation attenuated microvascular rarefaction after WBI

To evaluate the microvascular density of the brains of mice, we used immunofluorescence staining to detect the expression of vWF, which is a specific marker for endothelial cells. As shown in Fig. 7, the immunofluorescence intensity of vWF in the IR group was significantly reduced compared with the control group, indicating that irradiation-induced microvascular rarefaction occurred. After EPC transplantation, the vWF intensity was significantly increased compared to the irradiation group, which suggests that EPCs may enhance microvascular density.

2.6. EPC transplantation did not attenuate cognitive dysfunction after WBI

In order to test the learning memory of mice, we performed a Morris
water maze test 8 weeks after transplantation. The average escape latency among three groups at different timepoints were compared using a two-way repeated measures ANOVA. There was no significant interaction between the time and group (F = 0.226, p > 0.05). However, the main effects of time and group were significant. As shown in Fig. 8, the escape latency in the control group was significantly shorter than that in the IR group (P < 0.001). However, there was no significant difference between the EPCs group and IR group.

3. Discussion

Microvascular injury may cause either direct or indirect damage to parenchymal cells. It is well known that irradiation has a profound impact on the vasculature. Several studies have shown evidence of radiation-induced vascular changes, such as endothelial cell apoptosis, vascular wall thickening, vasodilation, increased vascular permeability, injury to the integrity of endothelial tight junctions, and decreased vascular density (Andrews et al, 2018; Yang et al, 2018).

Damage to the BBB and an increase in the BBB permeability are significant features of vascular injury after irradiation. The passage of most soluble molecules from the circulatory system to the central nervous system (CNS) is severely restricted by the BBB. When the BBB is destroyed, excessive extravasation of inflammatory cells and bioactive molecules can damage the brain. The BBB is composed of CNS endothelial cells, glial cells, and pericytes. It is thought that the integrity of the BBB depends mainly on tight junctions (Bednarczyk and Lukasiuk, 2011). Tight junction proteins are composed of zonula occludens 1, 2, and 3 (ZO-1, ZO-2, ZO-3), claudins, occludin, and other molecules that are key elements for BBB integrity (Obermeier et al, 2013). It has been shown that inducing the expression of tight junction proteins can enhance BBB recovery (Prakash and Carmichael, 2015).

BBB dysfunction is one of the most important pathophysiological factors for vascular injury following WBI. Thus, we investigated altered BBB integrity using intravitral two-photon imaging to measure BBB permeability and examined the expression of tight junctions in the brain. Our results showed that the leakage of 70 kDa Rhodamine B through the BBB increased after WBI, which indicates an increase in the BBB permeability. Since altered BBB permeability seems to be dependent on molecular size, this suggests damage to the integrity of endothelial tight junctions. We found that the expression of tight junction proteins decreased after WBI, suggesting damage to the BBB. Further, our previous study showed that ZO-1 gradually decreased with time from the first day to six months after WBI (Deng et al, 2017).

Decreased microvascular density is another important property of vascular injury following irradiation. Several studies have evaluated changes in endothelial cell function, as well as changes in the structure and density of cerebral vessels after irradiation. As endothelial cells are highly susceptible to irradiation, WBI can induce apoptosis of endothelial cells, repress endothelial cell proliferation, thicken the vascular basement membrane, disrupt the extracellular matrix, damage the
BBB (Mao et al., 2010; Zhou et al., 2017), and consequently lead to chronic microvascular rarefaction in the brain. Junie and colleagues reported that microvascular rarefaction occurs in the mouse hippocampus as early as 2 months following fractionated WBI (Warrington et al., 2011), which is consistent with our findings.

BBB disruption and microvascular rarefaction after WBI should induce mechanisms of vascular restoration to minimize long-term damage to the brain. Angiogenesis is considered to be the main mechanism responsible for the repair, maintenance, and development of vascular integrity after vessels are ruptured. EPCs play a vital role in angiogenesis by mobilizing from the bone marrow to lesions to stimulate vascular restoration (Caballero et al., 2007; Ribeiro et al., 2017). After vascular damage caused by WBI, EPCs circulate in the peripheral blood. Circulating EPCs are thought to express human stem cell markers (CD34 or CD133) and endothelial cell markers (VEGFR2, vWF, or CD31) (Samman et al., 2018). Research suggests that the CD34+ VEGFR2+ phenotype is important for EPCs function, laboratory detection, and is clinically significant (Fadini et al., 2012). This phenotype can be used to identify cells that are able to stimulate angiogenesis in vivo.

Reduction of circulating EPCs may lead to BBB disruption and microvascular density reduction after WBI. In the present study, flow cytometry demonstrated that WBI resulted in a significant decrease in circulating CD34+ VEGFR2+ EPCs compared with the control group. Therefore, bone marrow-derived EPCs were cultured and transplanted intravenously into mice who underwent WBI in order to increase the population and mobilization of EPCs in the circulatory system. Previous research has shown that culture-expanded EPCs significantly promote revascularization compared with fresh-isolated EPCs (Chong et al., 2016). In our research, EPCs were isolated and enriched by cultivation of MNCs which were collected from mouse bone marrow and then used for transplantation. The increased level of EPCs after transplantation may be due to its ability to proliferate (Asahara et al., 1997), and release cytokines including granulocyte colony stimulating factor, granulocyte–macrophage colony stimulating factor, vascular endothelial growth factor, etc. These cytokines can increase the recruitment of EPCs from the bone marrow to peripheral blood (Liu et al., 2009). Our results also indicated that BBB disruption and microvascular rarefaction were attenuated after transplantation. The extravascular Rhodamine B intensity decreased, and tight junction proteins increased compared with the IR group after EPC transplantation, indicating BBB recovery. Further, the microvascular intensity in the EPCs group was higher than that of the IR group.

The therapeutic effects of EPCs have been investigated in ischemic models. Medina reported that EPCs can closely interact with endothelial cells through adhesion and tight junctions, as well as integrate into retinal vascular networks after ischemic retinopathy (Medina et al., 2010a). It has also been shown that EPCs play a significant role in re-endothelialization of the injured blood vessels in atherosclerosis (Liu et al., 2009). The underlying mechanisms of how EPCs repair blood vessels have not yet been elucidated. However, it has been suggested that EPCs might contribute to vascular repair by direct cell replacement or trophic support to enhance the endogenous repair process. EPCs derived from the bone marrow have an intrinsic ability to differentiate into endothelial cells and replace the injured endothelium. In addition, an increasing number of studies show that EPC-mediated vascular protection may not entirely result from differentiation into endothelial cells. EPCs release angiogenic cytokines, including fibroblast growth factor, vascular endothelial growth factor, epidermal growth factor, transforming growth factor-β2, and platelet-derived growth factor, among others (He et al., 2004; Medina et al., 2010b). However, the trophic supporting effects of EPCs in the case of WBI requires further investigation.

In conclusion, continuous vascular dysfunction may be an important trigger for radiation-induced brain injury. Due to their angiogenic properties, EPCs can promote the reconstruction of the BBB after injury. We speculate that increased levels of circulating EPCs after WBI may restore damage to the BBB, tight junctions, and cerebral capillary density. Although the underlying mechanisms, as well as the optimal dose and timing of the EPC injection for clinical application requires further study, transplantation of EPCs may be a promising approach for the treatment of patients with RBI.

Fig. 3. Analysis of circulating endothelial progenitor cells (EPCs) levels. (A) Quantification of peripheral blood EPCs using flow cytometry. (a, b, c) Representative images of the blank and single-stained (CD34 or VEGFR2) cells. (d, e, f) Representative images of the percentages of CD34+ VEGFR2+ cells in the control, irradiation, and EPCs groups, respectively. (B) Comparison of the percentage of EPCs in the peripheral mononuclear cells (MNCs) among groups. n = 6. Data are presented as the mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001.
4. Experimental procedures

4.1. Animals

All procedures concerning animal treatment and care were conducted in accordance with the US National Institute of Health guidelines for the care and use of laboratory animals. The study was approved by the Ethics Committee on Laboratory Animal Care of Sun Yat-sen University (Guangzhou, China). We minimized the number of animals used and their suffering. Thirty-six male C57BL/6 mice were purchased and housed in a specific pathogen-free mouse room at the Experimental Animal Center of Sun Yat-sen University. The animals were maintained on a 12-hour light–dark cycle and had ad libitum access to water and food. When experiments began, the mice were 8 weeks of age. The mice were randomly divided into three groups, as follows: (1) control group (sham irradiation + PBS [phosphate buffer saline]), (2) irradiation (IR) group (WBI + PBS), and (3) the EPCs group (WBI + EPCs).

4.2. Whole-brain irradiation

WBI was administered using the RS2000 X-ray irradiator (Rad Source Technologies, Inc., USA). Mice in the IR and EPCs groups were anesthetized, and their heads were exposed to the irradiation field. A single dose of 10 Gy was administered at a rate of 1.22 Gy/min once a week, for 2 weeks. The mice in the control group underwent the same operation, but did not receive radiation.

Fig. 4. Measurement of Rhodamine B leakage using intravital two-photon imaging. (A) Representative image of the cranial window immediately after the injection of Rhodamine B (scale bar = 1 mm, 50 × ). (B) Representative images of the cerebrovascular and dye leakage at 5, 15, 30, 45, and 60 min after the injection (scale bar = 100 μm, 250 × ). (C) Line graph of the average intensity of extravascular Rhodamine B. n = 5. Data are presented as the mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001 (compared with the irradiation group).
Bone marrow-derived EPCs (BM-EPCs) were prepared as follows: bone marrow was collected from the femur and tibia of mice under sterile conditions. We isolated MNCs using density gradient centrifugation. Then, the cells were resuspended in EGM-2 Single-Quots (CC-4176, Lonza, USA) and incubated at 37 °C in 5% CO2 for 7 days to produce BM-EPCs. The medium was changed every third day.

The cell markers CD34, VEGFR2, CD133, and CD31 were detected by immunofluorescence microscopy. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min and then permeabilized by incubation with 0.5% Triton X-100 for 20 min before staining. Subsequently, cells were incubated at 4 °C overnight with primary antibodies against CD34, VEGFR2, CD133, or CD31, followed by corresponding secondary antibodies. Cells were observed under a magnification (×200) field using a fluorescence microscope. The EPCs were labeled by DiI (Beyotime, USA) for double-staining. Blank and single-staining served as controls.

For peripheral blood sampling, we clipped the tip of the mouse tail and collected 100 μL of blood from the tail 8 weeks after the WBI. Subsequently, the 100 μL samples were incubated for 30 min in the dark with the FITC-conjugated anti-mouse CD34 monoclonal antibody (1:50, 11-0341-85; eBioscience, USA) and with the PE-conjugated anti-mouse VEGFR2 (CD309) antibody (1:40, 12-5821-85; eBioscience, USA) for double-staining. Blank and single-staining served as controls. After incubation, cells were washed with PBS, lysed with a red blood cell lysis buffer (64010-00-100; Biogems, USA), and resuspended in PBS. Flow cytometry was performed with a BD AccuriC6 device (BD Biosciences, USA). For the FSC/SSC plots, MNCs were selected using a high FSC cell threshold which excluded small particles, residual granulocytes, and cellular debris. The CD34 and VEGFR2 dual-expressing population was quantified, and circulating EPCs were expressed as a percentage of CD34+ VEGFR2+ in peripheral MNCs.

The method of intravital two-photon imaging is described in our previous study (Zhou et al, 2019). Briefly, mice were anesthetized, and a cranial window with a diameter of 3 mm was made in the right hemisphere. We adhered a metal strip with a hole in the center to the mouse’s skull. Then, the metal strip was adhered to the plate to secure the mouse, which was then placed under a two-photon laser scanning microscope (Leica, Germany). In order to investigate the permeability of the BBB, Rhodamine B (70 kDa, R9379; Sigma-Aldrich, St. Louis, MO, USA) was injected intravenously to observe the cerebral vessels and to detect dye leakage. The red fluorescence channel was selected for detection. At 5, 15, 30, 45, and 60 min following the injection, the XYZ image stacks were collected to a depth of 100 μm (2 μm step size) below the cortical surface. We calculated the average fluorescence intensity in the extravascular space using Leica Application Suite Advanced Fluorescence 2.5 software.

Mice were euthanized 8 weeks after EPC transplantation. For immunofluorescence staining, mice were anesthetized and underwent intracardiac perfusion with normal saline followed by 4% PFA. The brains from each group were then removed, post-fixed in 4% PFA overnight, and then dehydrated with a sucrose gradient. For western blot analysis, mice were perfused with normal saline and the brains were quickly removed, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

The expression of tight junction proteins claudin-5 and ZO-1 were evaluated using western blotting. Brain tissue was harvested and placed in a mixture of RIPA buffer with a protease inhibitor on ice. The supernatant was separated by centrifugation at 12000 rpm for 30 min at 4 °C. We determined the total protein concentration in each sample using the BCA method (Thermo). Proteins (30 μg) were separated by SDS polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, USA). Membranes were blocked with TBST containing 5% nonfat dry milk for 1 h, and then incubated with a mouse anti-claudin 5 monoclonal antibody (1:1000, 352588; Invitrogen, USA) and a rabbit anti-ZO-1 polyclonal antibody (1:1000, AF5145; Affinity

4.3. Preparation and transplantation of EPCs

4.4. Quantification of EPCs in the peripheral blood by flow cytometry

4.5. Intravital two-photon imaging

4.6. Tissue sample preparation

4.7. Western blotting
Biosciences, USA) at 4 °C overnight. The bands were exposed to an antibody against GAPDH (1:2000, #2118; CST) to confirm equal protein loading. Then, the membranes were washed with TBST three times, and probed with corresponding HRP-conjugated secondary antibodies (1:3000, CST) at room temperature for 1 h. Finally, we used a western blot Imaging System (Amersham Imager 600, GE, USA) to detect the target protein. The intensity of the obtained band was quantified relative to the matching GAPDH band.

4.8. Immunofluorescence staining

We embedded the brains of mice in paraffin and sliced them with a cryostat microtome. Sections (10 μm thick) were sliced and mounted on glass slides. The sections were treated with immunostaining blocking buffer (P0102, Beyotime Biotechnology, China) at room temperature for 1 h, and subsequently incubated at 4 °C overnight with primary antibodies against claudin-5 (Alexa Fluor 488-conjugated mouse monoclonal antibody, 1:200, 352588; Invitrogen, USA), ZO-1 (goat polyclonal antibody, 1:200, sc-8146; Santa Cruz Biotechnology), and

![Fig. 6. Expression of tight junction proteins using immunofluorescence microscopy. (A) Representative images of zonula occludens-1 (ZO-1) expression. (B) Dot plot of the ZO-1 fluorescence intensity in all groups. (C) Representative images of claudin-5 (CLDN5) expression. (D) Dot plot of the claudin-5 fluorescence intensity. n = 6. Scale bar = 25 μm. Data are presented as the mean ± standard error of the mean. ***P < 0.001.](image)
vWF (mouse monoclonal antibody, 1:200, sc-365712; Santa Cruz), followed by secondary antibodies (FITC-conjugated anti-goat IgG, 1:400, sc-2356, Santa Cruz; and Alexa Fluor 555-conjugated anti-mouse IgG, 1:400, #4409; CST) for 1 h at room temperature. After nuclear staining with DAPI for 5 min, fluorescence images were obtained using a fluorescence microscope, and 6 random fields for each mouse were quantified.

### 4.9. Morris water maze

In order to test the learning memory of mice, we performed a Morris water maze test eight weeks after the transplantation. The water maze consisted of a round water tank (100 cm in diameter and 50 cm in height). A circular escape platform (diameter 6 cm), was immersed 1.5 cm below the water surface. The pool was divided into four quadrants (NE, SE, SW, and NW). The escape platform was located in the middle of the NW quadrant, and the position remained stable for five days. Mice were trained four times a day for 5 days. Each trial terminated once the mouse reached the platform within a cut-off time of 60 s, and the average escape latency of the four trials for each mouse was calculated. On the sixth day of the test, the platform was removed. Each mouse was placed into the SE quadrant and was allowed to swim for 60 s. The time spent in the target quadrant (NW quadrant) was recorded.

### 4.10. Statistical analysis

The immunofluorescent intensity and western blotting bands density were analyzed with ImageJ software. Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 20.0. We used a one-way analysis of variance (ANOVA) and follow-up LSD t-tests to compare the percentages of EPCs.
in peripheral blood, the immunofluorescent intensity, and western blotting bands density. The BBB permeability and escape latency were measured using a two-way repeated measures ANOVA. P-values < 0.05 were considered statistically significant.

CRediT authorship contribution statement

Xurui Huang: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft. Minping Li: Methodology, Validation, Investigation. Dongxiao Zhou: Methodology, Investigation, Data curation. Zhezhi Deng: Investigation, Data curation. Junjie Guo: Investigation, Data curation. Haiwei Huang: Conceptualization, Methodology, Supervision, Writing - review & editing.

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