Engulfment of platelets delays endothelial cell aging via girdin and its phosphorylation

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Abstract. Endothelial cells are critical in angiogenesis and maintain the homeostasis of the blood-brain barrier (BBB). Platelets (PLTs) are essential in vascular biology, including angiogenesis. The present study aimed to investigate the effect of PLTs on the aging of endothelial cells. Human brain microvascular endothelial cells (HBMECs) and human astrocytes were co-cultured to mimic the BBB. Transmission electron microscopy was used to observe the engulfment of PLTs. Confocal microscopy was used to observe the co-localization of PLTs, girders of actin filament (girdin) and phosphorylated (p-)girdin. Senescence-associated β-galactosidase (β-gal) staining, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and flow cytometry were performed to examine the cell senescence, viability and apoptosis, respectively. Transwell assays were performed to examine cell invasion and migration. Western blot analysis was performed to detect the expression of girdin, AKT and p-AKT. PLTs delayed senescence, and promoted the viability and resistance to apoptosis of the HBMECs. Cell invasion and migration were enhanced by PLTs. In addition, girdin and p-girdin were essential to the engulfment of HBMECs by PLTs. Mechanically, the inhibition of AKT signals reversed the effect of PLTs on HBMECs by increasing the activity of β-gal, decreasing the cell viability, and inhibiting the invasion and migration of the HBMECs. The engulfment of PLTs assisted in delaying the aging of endothelial cells via girdin and p-girdin, in which the AKT signal was involved. The present study indicated a potential strategy for delaying endothelial cell aging in the treatment of central nervous system diseases.

Introduction

The blood-brain barrier (BBB), as a membrane barrier in the central nervous system (CNS) that separates the brain from circulating blood, is vital to maintain neural microenvironment homeostasis and low permeability, which is closely associated with CNS disease (1). Endothelial cells are not only the primary components of the BBB, but they are also important in the BBB. Angiogenesis is critical in the formation of new blood vessels within this brain microvessel network (2). The initiation of angiogenesis and the formation of early vascular structures depend on endothelial cells (3), which are essential for maintaining the function and regulating the proliferation of vessels. Endothelial cells are also important in damage repair, ischemic adaptation and tumor formation (4,5). However, changes in the function and structure of the vascular wall occur with increasing age or through stimuli from other environmental factors, including hypertension, smoking and drinking. This leads to the compromised ability of endothelial cells in proliferation, migration and damage repair (6,7), which results in disrupted vascular hemostasis and vessel aging.

It is accepted that the dynamic reorganization of the actin cytoskeleton is important in cellular morphogenesis and motility. The phosphatidylinositol 3-kinase (PI3K)-AKT pathway exerts functions downstream of growth factors in facilitating endothelial cell growth, and its downstream targets are involved in vascular remodeling (8-10). Girders of actin filament (girdin), as an actin-binding protein in mammalian tissues, is expressed in limited cell types, including immature endothelial cells, which can be phosphorylated by AKT at serine 1417 (11,12). It has been reported that girdin is important in cell migration, which relies on extracellular signals and the surrounding microenvironment. Therefore, it is of value to delay the aging of endothelial cells and modulate the relevant molecule signaling for the prevention of CNS disease.

Platelets (PLTs), as fragments of cytoplasm derived from megakaryocytes, are components of the blood in mammals (13). The importance of PLTs in the inflammatory response has been reported. In addition, extensive evidence has revealed that PLTs are essential in vascular biology, including angiogenesis and tissue regeneration (14,15). Through various angiogenic stimulators, PLTs are capable of promoting de novo vessel growth. In the presence of vascular...
endothelial growth factor and platelet-derived growth factor, the proliferation of endothelial cells can be induced and their migration can be increased (16-18). Previously, attributable to concentrated biologically active molecules, platelet-rich plasma (PRP), as PLTs concentrate, has become more common as a clinical treatment to induce healing and regeneration, and control inflammation (19-21). Furthermore, exosome derived from PLTs has attracted wide attention owing to its role in angiogenesis and neovascularization (22,23). PLTs can be engulfed by endothelial cells (24), however, the precise effect of internalized PLTs on endothelial cells remains to be elucidated.

Multiple BBB in vitro models have been established, including monoculture, co-culture and triple culture systems. These systems have their respective advantages and disadvantages (25-27). In the present study, human brain microvascular endothelial cells (HBMECs) were used to mimic the BBB owing to its brain origin. Therefore, this model offers a more representative BBB model compared to several other cell lines currently used (26). In the present study, the effects of internalized PLTs on HBMEC senescence, proliferation, apoptosis, invasion and migration were determined, and the associated mechanisms were established in vitro. This information may provide a novel method for delaying the aging of endothelial cells, inspiring a potential strategy for angiogenesis in the treatment of CNS diseases.

Materials and methods

Cell culture and treatment. The HBMECs (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured with endothelial cell medium (ScienCell Research Laboratories) containing 10% fetal bovine serum (FBS) and 1% endothelial cell growth supplement (ScienCell Research Laboratories) in a 5% CO2 humidified incubator at 37°C. The HBMECs (5x104 cells per cm2) were seeded onto the collagen/fibronectin-coated Transwell insert. Subsequently, human astrocytes (HAS) (ScienCell Research Laboratories) were seeded (5x104 cells per cm2) onto the carrier plates in the Transwell and cultured with astrocyte medium (ScienCell Research Laboratories). The medium was replaced with fresh medium every other day. After 3 days, the insert was coated with HBMECs was added to dishes containing the HAS. Lipofectamine 3000 (cat. no. L3000008; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for cell transfection. Short hairpin (sh)-girdin plasmid (cat. no. sc-94984-SH) and control shRNA plasmid (cat. no. sc-108060) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mutated girdin was transfected into HBMECs. Short hairpin (sh)-girdin plasmid (cat. no. sc-94984-SH) and control shRNA plasmid (cat. no. sc-108060) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mutated girdin was transfected into HBMECs. Lipofectamine 3000 (cat. no. L3000008; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for cell transfection. Short hairpin (sh)-girdin plasmid (cat. no. sc-94984-SH) and control shRNA plasmid (cat. no. sc-108060) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mutated girdin was transfected into HBMECs. Short hairpin (sh)-girdin plasmid (cat. no. sc-94984-SH) and control shRNA plasmid (cat. no. sc-108060) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mutated girdin was transfected into HBMECs.

β-galactosidase (β-gal) staining. The activity of senescence-associated (SA)-β-gal was detected with a β-gal staining kit (cat. no. C0602; Beyotime Institute of Biotechnology, Haimen, China). Briefly, the cells were fixed with 0.2% glutaraldehyde solutions for 5 min, followed which the cells were stained with X-gal solution for 18 h at 37°C. The SA-β-gal-positive cells were observed under a phase-contrast microscope at x100 or x400 magnification (Olympus, Tokyo, Japan). The proportions of cells positive for SA-β-gal activity were determined as a percentage of the total number of cells counted in each dish.

Flow cytometry for detection of the engulfment of PLTs. The engulfment of fluorescent PKH26-labeled PLTs by endothelial cells was determined using flow cytometry. Briefly, the cells were incubated in HEPES-buffer, comprising 134 mM NaCl, 6 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES and 10 mM glucose (pH 7.40), and anti-PLT antibody (AIP21; cat. no. ab112238; Abcam, Cambridge, MA, USA; 100 nM) for 30 min at 4°C, followed by a 15-min wash with 1X PBS buffer. Flow cytometric analysis (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze the mean fluorescence intensity.

Flow cytometric analysis of apoptosis. Apoptosis was determined using an Annexin V/PI apoptosis kit (MultiSciences Biotech Co., Ltd., Hangzhou, China). The cells were harvested and centrifuged at 2,000 x g at 4°C for 5 min. Following collection, the cells were resuspended in 0.5 ml 1X annexin-binding buffer at a density of 5x105 cells/ml. Annexin V-FITC and PI were added and the cells were incubated for 10 min at room
temperature in the dark. The samples were immediately
analyzed by flow cytometry.

Transmission electron microscopic observation. In brief, the
cells were fixed in 4% glutaraldehyde/1% paraformaldehyde
for 2 h at room temperature, and then post-fixed in 1% OsO₄
for 1 h at 4˚C. Following dehydration in graded ethanol
(70, 95 and 100%), the cells were embedded in LX112.
Ultrathin sections (50 nm) were stained with 1% uranyl acetate
and lead citrate, and were then examined for mitochondrial
morphology on a H7000 electron microscope operating at
80 kV (Hitachi, Ltd., Tokyo, Japan).

Confocal microscopy. The cell sections were incubated
with 3% hydrogen peroxide for 10 min. Normal goat serum
(Beijing Solarbio Science & Technology Co., Ltd., Beijing,
China) was added to the sections and incubated at room
temperature for 2 h. Subsequently, the sections were incubated
with anti-PLT antibody (100 nM; cat. no. ab53304; Abcam),
anti-girdin antibody (1:500; cat. no. ab179481; Abcam), or
anti-girdin (phosphorylated S1417) antibody (8 µg /ml; cat.
no. 28067; Takara Bio, Inc., Otsu, Japan) at 4˚C overnight.
The secondary antibodies, FITC-donkey anti-rabbit IgG
(1:200; JacksonImmuno Research Laboratories, Inc., West
Grove, PA, USA) and anti-rabbit IgG (H+L) F(ab')2 Fragment
(1:500; Alexa Fluor® 488 Conjugate; cat. no. 4412; Cell
Signaling Technology, Inc., Danvers, MA, USA) were added
and incubated at 4˚C overnight. Finally, the sections were
mounted with FlourSave (calbiochem; Merck Millipore)
mounting reagent. Images of the histological sections
were captured using Northern Eclipse software version 6.0
(Empix Imaging, Inc., Mississauga, ON, Canada) on a Zeiss
Axioplan 2 imaging microscope (Carl Zeiss, Toronto, ON,
Canada).

Transwell assay. A precoated cell invasion kit (pore size
8.0-µm; Corning Costar) and Matrigel (250 µg /ml; BD Biosciences) in Transwell chambers were used in Transwell
assays. The cells were plated at a density of 5x10⁴/ml in the
upper chambers without FBS. The cells migrated to the lower
chamber containing medium with 30% FBS. The invaded cells
were fixed with paraformaldehyde after 60 h incubation and
stained with 0.1% crystal violet. The numbers of cells that
invaded through the membrane were counted under a light
microscope (Nikon Ni-E; Nikon corporation, Tokyo, Japan) in
four randomly selected fields per well.

Western blot analysis. The samples were incubated with an
NP40 lysis buffer (Beijing Solarbio Science & Technology
Co., Ltd.) with protease inhibitors (Promega Corporation,
Madison, WI, USA). Then the concentration of the protein
samples was estimated using a bicinchoninic acid protein
quantitation kit (Thermo Fisher Scientific, Inc.). Protein extract
(35 µg per group) was separated on 8% SDS-polyacrylamide
gels and transferred onto a nitrocellulose membrane. The
membrane was blocked in 5% skim milk powder solution

Figure 1. Phagocytosis of PLTs mitigates cell aging. (A) Cell senescence was determined by SA-β-gal staining (magnification, x100) and (B) quantified. (C) Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *P<0.05 and **P<0.01. PLT, platelet; SA-β-gal, senescence-associated β-galactosidase.
with 0.1% Tris-buffered saline/Tween 20 at room temperature for 2 h. The membrane was then incubated with anti-active caspase-3 antibody (1 µg/ml; cat. no. ab2302; Abcam), anti-AKT1/2/3 (phospho Y315+Y316+Y312) antibody (1:500; cat. no. ab131443), anti-pan-AKT antibody (1 µg/ml; cat. no. ab85683), anti-girdin antibody (1:40; cat. no. 18979; Takara Bio, Inc.), anti-girdin (phospho S1417) antibody (1:50; cat. no. ab8245; Abcam) overnight at 4˚C. The target bands were detected using a horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. ab6721; Abcam) by incubation for 1 h at room temperature and then visualized using a BeyoECL plus enhanced chemiluminescence detection system (Beyotime Institute of Biotechnology). The band intensity was normalized to that of GAPDH and expressed as a relative ratio.

Statistical analysis. Statistical analysis was performed with SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). For comparisons between groups, Student’s t-test or one-way analysis of variance followed by Dunnet’s multiple comparison test was used. P<0.05 was considered to indicate a statistically significant difference.

Results

PLTs promotes resistance to cell senescence. The expression of SA-β-gal can be induced in senescent cells. As shown in Fig. 1A and B, the activity of SA-β-gal was reduced by PLT treatment. Cytochalasins can alter cell morphology by inhibiting actin polymerization. Following treatment with cytochalasin D, the decline in activity was recovered partially. In addition, the cell viability was increased in the PLT group; and was decreased by cytochalasin D (Fig. 1C).

PLTs depress the apoptosis of HBMECs. The proliferation of cells can be inhibited by senescence or apoptosis. The apoptosis of HBMECs was determined in the present study. The results revealed that the level of apoptosis was decreased significantly in the PLT group, compared with that in the control group. By contrast, the number of apoptotic cells was higher in the cytochalasin D+PLT group, compared with that in the PLT group (Fig. 2A and B).
addition, the expression of cleaved caspase-3 was suppressed in the PLT group, compared with the control. Cytochalasin D recovered the protein level of cleaved caspase-3 (Fig. 2c and d).

**PLTs enhance the invasion and migration capability of HBMECs.** Invasion and migration are the essential motilities of endothelial cells (30). The Transwell assays revealed that the invasion and migration abilities of the cells were increased by PLTs, whereas cytochalasin d repressed the cell invasion and migration induced by PLTs (Fig. 3A-d).

**Effect of girdin on the engulfment of HBMECs to PLTs.** The transmission electron microscopy showed that, compared with the control group, knockdown of the expression of girdin inhibited the engraftment of endothelial cells to PLTs; and the mutated girdin at the 1417 locus also inhibited the engraftment of PLTs (Fig. 4A). In addition, the fluorescent intensity of PLT-labeling was reduced by the knockdown of girdin or the mutated girdin, compared with that in the PLT group (Fig. 4B and C). The downregulation of girdin was confirmed by western blot analysis (Fig. 4D). Furthermore, the engulfment of PLTs promoted the expression of girdin and p-girdin; whereas the expression of p-girdin was inhibited by the knockdown of girdin, while the expression of p-girdin did not appear to be influenced by m-girdin (Fig. 5A and B).

**Girdin is co-located with PLTs in HBMECs.** An immunofluorescence assay was performed to confirm the effect of girdin on the engulfment of PLTs. As shown in Fig. 6, girdin and p-girdin were located in the Golgi apparatus, and green fluorescence indicated phagocytic PLTs. Compared with the PLT group, the knockdown of girdin or the mutated girdin prevented the engulfment of PLTs.

**Akt signals are involved in the engulfment of PLTs.** It has been reported that girdin is a substrate of AKT/PKB, which is reported to be positively associated with phagocytosis (31). Triciribine, a specific inhibitor of AKT phosphorylation (32), was used in the present study to further determine the involvement of AKT. The inhibition of senescence of HBMECs caused by PLTs was reversed by triciribine (Fig. 7A). In addition, the cell invasion and migration capabilities were decreased by triciribine, compared with those in the PLT group (Fig. 7B-D). Mechanically, p-AKT was increased in PLT group, whereas treatment with triciribine significantly inhibited the activity of p-AKT.
Figure 4. Girdin is essential for the engulfment of PLTs. (A) Engulfment in HBMECs of PLTs was observed under a transmission electron microscope. Scale bar=5 µm. (B) Flow cytometric analysis of the engulfment of PLTs with (C) quantification. (D) Transfection efficiency of the sh-girdin vector was detected using western blot analysis. *P<0.05 and **P<0.01. PLT, platelet; HBMECs, human brain microvascular endothelial cells; sh, short hairpin RNA; m, mutated.

Figure 5. Expression of girdin and p-girdin are increased by PLTs. (A) Images and (B) relative intensity of western blot analysis to examine the expression of girdin and p-girdin. *P<0.05 and **P<0.01. PLT, platelet; p-, phosphorylated; sh, short hairpin RNA; m, mutated.
of AKT (Fig. 8A and B). This indicated that the activation of AKT signals was involved in the engulfment of PLTs.

Discussion

BBB is a well-differentiated microvessel network in the CNS and important to the hemostasis of brain tissues. Brain angiogenesis is a critical step in the development of the BBB, which is dependent on the invasion of endothelial cells into the avascular neuroectoderm and formation of intraneural vessels (33,34). It is promising for the protection of endothelial cells from injury and aging. In the present study, co-cultures of HAs and HBMECs in vitro were used to mimic the BBB in living cells. It was shown that PLTs promoted the resistance to senescence of HBMECs, whereas this effect was mitigated by treatment with cytochalasin D. Although the effect of extracellular PLTs cannot completely excluded, it was suggested that the survival of HBMECs was partly promoted by the phagocytosis of PLTs. The apoptosis of HBMECs was further examined, and the results revealed that the level of apoptosis was significantly decreased by PLTs. In addition, the expression of cleaved caspase-3, an apoptosis-associated protein (35), was inhibited by PLTs. These results indicated that treatment with PLTs increased proliferation and depressed endothelial apoptosis. This was consistent with previous reports that PLTs enhance the growth and integrity of endothelial cells (36,37). The invasion and migration of endothelial cells is an important event in angiogenesis (38). To further confirm the effect of PLTs, a Transwell assay was performed. It was shown that the PLTs augmented the invasion and migration abilities of the endothelial cells. Taken together, treatment with PLTs was beneficial for the healthy situation of endothelial cells. Consistently,
Figure 7. Activation of AKT facilitates the effect of PLTs on HBMECs. (A) Inhibition of AKT increased the activity of β-gal (magnification x400). (B) Inhibition of AKT depressed the (C) invasion and (D) migration of HBMECs (magnification, x100). **P<0.01 and ***P<0.001. PLT, platelet; HBMECs, human brain microvascular endothelial cells; SA-β-gal, senescence-associated β-galactosidase; Tri, triciribine.

Figure 8. Activity of AKT is increased by PLTs. Expression levels of AKT and p-AKT were (A) visualized and (B) measured using western blot analysis. **P<0.01 and ***P<0.001. PLT, platelet; Tri, triciribine; p-, phosphorylated; t-, total.
several studies have confirmed that PRP exerts protective effect via anti-inflammatory and anti-apoptotic effects, and by promoting angiogenesis (20,39). However, platelet aggregation and abnormal angiogenesis are considered to be harmful to human health (40–42). The concentration used in the present study may not be the optimal concentration for delaying cell aging, and further investigations are warranted for its potential clinical use. It is important to regulate the phagocytosis of PLTs in an appropriate manner.

PLTs can be engulfed by endothelial cells (24,29). The phagocytosis of PLTs was observed under a transmission electron microscopic. It was shown that the PLTs were engulfed by HBMECs. Girdin, as an actin-binding AKT substrate, is able to regulate angiogenesis (12). Girdin is also important for the migration of endothelial cells during postnatal angiogenesis (11,12). Therefore, it is possible that girdin may be associated with the protective effect of PLTs on endothelial cells. As expected, the depletion of girdin and mutated girdin (resistant to phosphorylation at 1417) suppressed the phagocytosis of PLTs by HBMECs. In addition, the expression of girdin and p-girdin were induced by PLTs. The co-localization of PLTs, girdin and p-girdin further demonstrated the potential role of girdin in the positive effect of PLTs on endothelial cells. As the reduction of girdin and p-girdin reduced the phagocytosis of PLTs, the depletion of girdin may have a negative feedback effect on the phagocytosis in endothelial cells of PLTs.

In consideration of the regulatory association between girdin and AKT, the involvement of AKT signals in the present study model was investigated using its specific inhibitor. The results confirmed that the repression of AKT signals reversed the effect of PLTs through the aggravation of senescence and the inhibition of invasion and migration of HBMECs. It was shown that phagocytosis of the PLTs induced the activation of AKT signals and thus contributed to cell growth and survival. This result was in line with previous reports (36,43). In addition, girdin can be phosphorylated by AKT at serine 1417. Therefore, the phosphorylation of girdin mediated by AKT may be the mechanism by which phagocytosis of PLTs promoted the survival of endothelial cells. This result was compatible with a previous study that highlighted the major effect of girdin and its Akt-mediated phosphorylation on migration and proliferation following vascular injury (19). Girdin is also associated with Wnt signaling pathways (44), indicating the versatile function of girdin in different signaling pathways. The intracellular signaling pathways are complex and there may be cross-talk, therefore, it is possible that other signals or pathways are involved in the phagocytosis of PLTs. For example, forkhead box 3a (Fox3a), another downstream target of the AKT signal, has been shown to be essential in angiogenesis (45); additionally, the extracellular signal-regulated kinase (ERK) pathway is another important signaling pathway for cell survival, angiogenesis and phagocytosis (46–48). Although the role of other signals, including the Fox3a and ERK pathways, were not elucidated in the present study, they may be modulated following the phagocytosis of PLTs and may be potential targets of novel therapeutics for delaying the aging of endothelial cells.

In conclusion, it was shown that the engulfment of PLTs delayed endothelial cell aging via the promotion of cell proliferation and inhibition of apoptosis, and the enhancement of cell invasion and migration abilities. Girdin was essential for the phagocytosis of PLTs, in which AKT may be involved in the phosphorylation of girdin. The results of the present study indicated the significance of understanding the effect of PLTs on endothelial cells, which may assist in developing novel therapeutics for CNS diseases associated with the aging of endothelial cells.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YLa, YoLi and DL wrote the manuscript. YLa, YoLi, DL, PL, JW, YD and GY performed the experiments. YLa and YaLi designed the study. YLa, YoLi, DL, PL and JW performed the data analysis. YLa, YoLi, and DL revised the manuscript and all authors reviewed the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Beijing Hospital (Beijing, China). Informed consent was obtained from the volunteers.

Consent for publication

Informed consent was obtained from all participants for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

References


