Exosomes from Adipose-Derived Mesenchymal Stem Cells Overexpressing Stanniocalcin-1 Promote Reendothelialization after Carotid Endarterium Mechanical Injury

Kun Liu
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Huihua Shi
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Zhiyou Peng
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Xiaoyu Wu (✉ wendaoliu1984@163.com)
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Weimin Li
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Xinwu Lu
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Research

Keywords: adipose-derived mesenchymal stem cells (ADSC), Stanniocalcin-1, mice arterial endothelial cells (MAEC)

DOI: https://doi.org/10.21203/rs.3.rs-115771/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background and objective:** Endothelial cell inflammation caused by mechanical injury of endovascular treatment remains the major obstacle to reendothelialization, which leads to arterial restenosis. We investigated the reendothelialization effect of exosomes from adipose-derived mesenchymal stem cells (ADSC) overexpressing Stanniocalcin-1 (STC-1).

**Methods:** Primary ADSCs were extracted from the adipose tissue of the inguinal area of C57/BL mice. ADSCs were transfected with lentivirus vectors containing STC-1. Exosomes were purified from culture medium using the Exo-Quick kit and characterized by transmission electron microscopy, nanoparticle tracking analysis and western blot. PHK-26 as molecular probe was used to track the exosomes engulfed by mice arterial endothelial cells (MAEC). The role of STC-1-ADSC-Exosome (S-ADSC-Exo) in MAECs was verified through scratch test and tube forming experiment. Carotid endarterium mechanical injury was induced by insertion with a guidewire into the common carotid artery lumen. Exosomes were administered by tail vein injection. Content of Reactive oxygen species (ROS) was measured using commercial kits. Carotid arteries were harvested for histological examination, immunofluorescence staining, and Evan's blue staining.

**Results:** Transfection of STC-1 significantly enhanced STC-1 levels in ADSCs, their exosomes, and MAECs. Compared with the control group and the ADSC-Exo group, STC-1 enriched exosomes markedly enhanced STC-1 level, inhibited the expression of NLRP3, Caspase-1, and IL-1β in MAECs, exhibited good lateral migration capacity, and promoted angiogenesis. Exosome-pretreating groups exhibited lower levels of ROS than those of controls. In vivo administration of S-ADSC-Exo had reendothelialization effect on post-injury carotid endarterium as evidenced by thinner arterial wall, low-expressed NLRP3, and more living endothelial cells.

**Conclusions:** The reendothelialization effect of exosomes from ADSCs on post-injury carotid endarterium can be enhanced by genetic modification to contain elevated STC-1.

Introduction

Approximately 18–25% ischemic strokes are caused by extracranial carotid atherosclerotic stenosis [1]. For patients at high risk of surgical complications, combination carotid angioplasty with stent implantation has become an accepted alternative to endarterectomy [2]. Despite refinements of interventional technique, the mechanical injury to carotid endarterium is still unavoidable completely and subsequent inflammatory restenosis is not conducive to ensure the long-term patency [3]. Post-injury restenosis just like wound-healing comprises complex pathophysiological mechanisms consisting of inflammation, proliferation, and migration followed by remodeling of arterial wall [4]. Persistent and exaggerated inflammation triggers the release of numbers of cytokines and growth factors, resulting in hyperplasia of neointima and proliferation of smooth muscle cells [5]. Therefore, inhibition of
inflammation, restoration of endothelial cell function, and promotion of reendothelialization are effective approaches to prevent and treat restenosis after endovascular treatment.

Exosomes carry a wide range of bioactive molecules, such as DNAs, mRNAs, microRNAs, cytoskeletal elements, proteases, signaling molecules and play important roles in intracellular information communication [6, 7]. Bioactive molecules can be transported to target cells under physiologic and pathologic conditions, which induce functional and expressional changes [7]. Recently, with regard to the role in the process of inflammation, exosomes derived from different cell types have drawn much interest. Via exosomes, IL-4 modified macrophages foster M2 polarization and inhibit inflammation to retard atherosclerosis through transferring their regulatory microRNAs to target cells [8]. Exosomes generated from cardiomyocytes and endothelial cells followed acute myocardial infarction are taken up by macrophages and regulate local inflammation to attenuate ischemic injury [9]. Mesenchymal stem cells (MSC) are multipotential stem cells that can promote immunomodulation, angiogenesis, matrix remodeling in the injured vessels by secreting factors to activate the signaling pathways involved in vascular repair. The powerful paracrine capacity of MSCs, not their differentiation potential, is the principal mechanisms of their repair action [10]. Local transplantation of adipose-derived mesenchymal stem cells around arteriovenous fistula alleviates restenosis and restores patency after PTA in mice [11]. MSCs have been reported to exert their powerful immunomodulatory and anti-inflammatory effects in the pathological process of atherosclerosis [12]. However, the microenvironment of target tissue may affect ADSCs survival and migration, which leads to poor long-term prognosis [13]. Compared with other cell types, MSCs are more likely to secrete a great quantity of exosomes, and exosomes can partially overcome the above deficiencies due to their low immunogenicity [14]. Umbilical cord mesenchymal stem cell-derived exosomes have been indicated to attenuate TNF-α induced inflammation in endothelial cells [15]. Mesenchymal stromal cell-derived exosomes are shown to ameliorate diabetic peripheral neuropathy by suppression of proinflammatory genes [16]. With the development of exosome research, it has been found that the function of exosomes can be enhanced by genetic modification of stem cells to load target proteins [17].

Interleukin (IL) -1β has been reported to participate in the chronic inflammation and restenosis after endovascular treatment [11, 18]. As an important component of innate immunity, NLRP3 inflammasome consisting of NLRP3 receptor protein, apoptosis-associated speck-link protein containing a CARD (ASC), and Caspase-1 has recently emerged as a protein complex for inducing the release of IL-1β and IL-18[19]. ROS are key molecule signal for activating NLRP3[20], and proved to be triggered after stent implantation and PTA [21]. STC-1 is a kind of glycoprotein directly act on mitochondria and then inhibit ROS through uncoupling [22]. Thus, it is plausible that the axis of STC-1-NLRP3- IL-1β may be the new target for protecting endothelial cells against inflammation induced by mechanical injury and promoting reendothelialization. In the current study, we delivered STC-1 to endothelial cells by exosomes from ADSCs that were genetically modified to overexpress STC-1. We highlight the reendothelialization effect of STC-1-modified exosomes on post-injury carotid endarterium in the setting of inflammation caused by mechanical injury.
Materials And Methods

Animals

Male, 6-8 weeks C57/BL mice were enrolled in the present study. The animal protocol was approved by the Ethics Review Committee of Animal Experimentation of Shanghai Jiao Tong University School of Medicine (Shanghai, People's Republic of China).

Isolation, culture, identification and transfection of ADSCs

The primary ADSCs were extracted from the adipose tissue of the inguinal area of C57/BL mice. The harvested adipose tissues were digested with NB4 collagenase (Nordmark, Uetersen, German) in low glucose (1000 mg mL\(^{-1}\)) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, U.S.A). After 60 min digesting and shaking (220 rpm) at room temperature, the cells were resuspended and seeded on dishes, and used at passage 3. To identify the phenotype of ADSCs, the specific surface markers were determined by flow cytometry analysis (Beckman Coulter, Fullerton, CA). The mouse antibodies against CD29, CD31, CD34, and CD44 were conjugated with FITC. ADSCs were incubated with the above antibodies in 100 mL FACS buffer (Sigma, San Francisco, U.S.A). The labeled cells were washed three times with 1.5 mL FACS buffer and fixed with cytofix (BD Pharminogen, San Francisco, U.S.A). The Beckman Coulter flow cytometer and the FlowJo software (Version 10.0) were used to analyze data. Mice STC-1 lentivirus gene transfer vectors were constructed by Genechem (Shanghai, China). 1´10\(^6\) ADSCs were seeded in 10 mL of low glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin overnight and were subsequently transfected with 200nM STC-1 vector at a multiplicity of infection of 10 for 24 hours. After that, the envelope suspension was transferred to normal culture medium, and the transfected ADSCs were cultured for 48 hours. The green fluorescent protein (GFP) encapsulated in lentivirus vectors was observed using fluorescence microscope.

Exosomes extraction and identification

At 48 hours after transfection, the exosomes were isolated at 4°C according to the method previously described[23]. Firstly, the culture medium of ADSCs was filtered to remove large debris and floating cells. Secondly, small debris was removed by centrifugation at 10,000´g for 30 min. Lastly, the remaining supernatants were further centrifugated at 10,000´g for 3 h. The precipitate was resuspended in phosphate-buffered saline (PBS) and the diameters of exosomes were measured using nanoparticle tracking analysis. The morphologic characteristics of exosome were observed using transmission electron microscopy (TEM). Expression of CD9, CD63 and CD81, as markers of exosomes, were confirmed by Western blot.

Mice arterial endothelial cells (MAECs) culture and tracking of exosomes engulfed by MAECs
MAECs were purchased from Daixuan Biosciences Inc (Shanghai, China) and cultured in high glucose (4500 mg mL\(^{-1}\)) DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. MAECs were cocultured with PHK26 labelled exosomes for 24 h. The exosomes engulfed by MAECs were identified by fluorescence microscopy. DAPI was used to stain nucleus, and Phalloidin was used to stain cytoskeleton.

**NLRP3 inflammasome activation**

NLRP3 inflammasome was activated according to the method previously described [22]. MAECs (1´10\(^5\)) were seeded in 6-well plates. The culture medium was added with LPS (Ultra-pure InvivoGen, San Diego, CA) at the concentration of 2 mg mL\(^{-1}\) in high glucose DMEM for 6 hours. After washing with PBS three times, the MAECs were incubated with 5 mM ATP (InvivoGen, San Diego, CA) for 45 min. After that, the MAECs were washed with PBS for three times and cultured in high glucose DMEM. Scraping with a pipette tip was conducted in the upper 3 wells of 6-well plate, with the lower 3 wells receiving no procedure as control. 24 hours later, the cells were collected to determine the expression of NLRP3 inflammasome.

**Scratch test and Tube forming experiment**

The lateral migration capacity of MAECs was assessed by scratch test. 1´10\(^5\) MAECs were seeded in 6-well plates and scraped by a pipette tip to generate uniform wounds when reached at approximately 90% confluent. Each well was washed three times with PBS to remove floating cells, and then the MAECs were cultured in medium with no serum. The blank area at the time intervals (0 h, 6 h, 12 h, and 24 h) were observed under inverted microscope and calculated using Image-Pro Plus 6.0 software.

10 mL of Matrigel per well was evenly placed on the angiogenic slide (Ibidi, Germany). After that, the slide was placed in an incubator for 2 hours to solidify the Matrigel. About 1´10\(^4\) MAECs were seeded on per well and observed under inverted microscope after 4-6 hours.

**Animal model of carotid endarterium wire-injury and tail vein injection**

After administering anesthesia (pentobarbital sodium, 0.5 mg g\(^{-1}\)), the mice were fixed on a heating plate maintained at 37 °C. A middle neck incision was performed, the left common carotid artery and its branches were skeletonized. After dissection, mechanical injury to carotid endarterium was induced by insertion with a guidewire into the left common carotid artery. At the end of procedure, the guidewire was removed, the proximal and distal sutures were tied off gently. The procedure is shown in Fig. S1. The administration of exosomes was performed through tail vein injection.

**Experimental protocol**

Experimental groups and protocol are described as followed.

**In vitro.** To evaluate angiogenic effect of STC-1 overexpressing exosomes on MAECs after scrapping, 3 groups were enrolled. The NLRP3 inflammasome was activated by addition of LPS + ATP according to
the method as described above. The scratch test: Control group: PBS (2 mL) was added to the 6-well plate cultured with MAECs (1´10^5 per well) after scraped by a pipette tip. ADSC-Exo group: ADSC-Exo (2 mL, 2mg mL^{-1}) was added to the 6-well plate cultured with MAECs (1´10^5 per well) after scraped by a pipette tip. S-ADSC-Exo group: S-ADSC-Exo (2 mL, 2mg mL^{-1}) was added to the 6-well plate cultured with MAECs (1´10^5 per well) after scraped by a pipette tip.

The tube forming experiment: Control group: PBS (2 mL) was added to the angiogenic slide cultured with MAECs (1´10^4 per well). ADSC-Exo group: ADSC-Exo (2 mL, 2mg mL^{-1}) was added to the angiogenic slide cultured with MAECs (1´10^4 per well). S-ADSC-Exo group: S-ADSC-Exo (2 mL, 2mg mL^{-1}) was added to the angiogenic slide cultured with MAECs (1´10^4 per well).

In a parallel series of experiments, MAECs were collected from the 3 groups 24 hours after scratching to determine the expressions of NLRP3 inflammasome.

**In vivo.** To evaluate the reendothelialization effect of STC-1overexpressing exosomes on post-injury carotid endarterium in the wire-injury model, 3 groups were enrolled. Control group (n=10): PBS (100 mL) was injected through caudal vein 1 hour after left common carotid artery wire-injury. ADSC-Exo group (n=10): ADSC-Exo (100 mL, 2mg mL^{-1}) was injected through caudal vein 1 hour after left common carotid artery wire-injury. S-ADSC-Exo group (n=10): S-ADSC-Exo (100 mL, 2mg mL^{-1}) was injected through caudal vein 1 hour after left common carotid artery wire-injury. All the mice were sacrificed 14 days after operation, and left common carotid arteries were harvested for histological examination, immunofluorescence staining, and Evan's blue staining.

In a parallel series of experiments, the left common carotid arteries were additionally harvested from the 3 groups (n=5 per group) to evaluate the expressions of NLRP3 inflammasome.

**Enzyme-Linked Immunosorbent Assay**

Contents of ROS were measured using enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions (Thermo Fisher, Shanghai, China).

**Histological examination**

On the 14th day after procedure, the post-injury carotid arteries were harvested for histological analysis. Paraffin-embedded sections (4 mm) of left common carotid arteries were stained with hematoxylin-eosin (HE) dye to determine the thickness of arterial wall. Immunofluorescence staining of the left common carotid arteries was performed to determine the expression of NLRP3 inflammasome. To determine the reendothelialization effect on post-injury carotid endarterium, 1% Even's blue dye was infused into the carotid arteries to delineate the injured area from reendothelialized area.

**Quantitative real-time polymerase chain reaction**
Total RNA of ADSCs, exosomes, and MAECs was isolated with Trizol reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer’s instruction. Quantitative real-time polymerase chain reaction was conducted according to a standard protocol with the Step One Plus system (Applied Biosystems, Foster City, Calif). GAPDH was used as internal control. Expressions were determined by $2^{\Delta\Delta CT}$ method.

**Western blot**

STC-1 in ADSCs after transfection, CD9, CD63, CD81, and STC-1 in their exosomes and NLRP3, Caspase-1, and IL-1β in MAECs were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with anti-STC-1, anti-CD9, anti-CD63, anti-CD81, anti-NLRP3, anti-Caspase-1, and anti-IL-1β antibodies (Abcam, Cambridge, Mass). Expressions of these proteins were analyzed with NIH Image (Research Services Branch, National Institutes of Health, Bethesda, Md) and quantified as relative folds to the control group after normalization with GAPDH.

**Statistical analysis**

Parametric values were analyzed by one-way analysis of variance followed by Bonferroni correction for post hoc testing. $P < .05$ was considered statistically significant. Statistical analysis was performed with SPSS, version 25.0 (IBM-SPSS Inc, Armonk, NY).

**Results**

**Characterization of ADSCs**

ADSCs extracted from C57/BL mice at passage 3 (Fig. 1a) were used in the following experiments. Flow cytometry analysis showed that specific surface antigens of stem cell (CD29 and CD44) were strongly positive, while the specific surface antigens of hematopoietic cell (CD31 and CD34) were negative (Fig. 1b).

**Fig. 1** The characterization of ADSCs. **a** Representative image of ADSCs in passage 3. **b** Flow cytometry analysis of ADSCs showing strongly positive expression of CD29 and CD44, while negative expression of CD31 and CD34. Scale bar = 100 mm

**Transfection with STC-1 lentivirus vectors and exosome characterization**

After transfection with STC-1 lentivirus vectors, GFP encapsulated within lentivirus vectors made ADSCs present green fluorescence (Fig. 2a). Exosomes derived from ADSCs were membrane vesicles (Fig. 2b). The result of nanoparticle tracking analysis showed that 99% of exosomes concentrated on 193 nm in diameter (Fig. 2c). The surface markers CD9, CD63, and CD81 of exosomes were confirmed by western blot (Fig. 2d).

**Fig. 2** Transfection and exosome characterization. **a** Fluorescent image of STC-1 transfected ADSCs. **b** Electron microscopic image of exosomes. **c** The diameter of exosomes ranges from 14.9 nm to 1036.5
nm, and 99% of them are 193 nm in diameter. d Western blots showing expressions of CD9, CD63, and CD81 in exosomes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Scale bar = 200 mm

**PHK26 labeled ADSC-Exosomes engulfed by MAECs**

The PHK26 labeled exosomes could be engulfed and detected in MAECs (Fig. 3). Fluorescence microscopy demonstrated that PHK-26 labeled exosomes were stained (red), with nuclear staining with DAPI (blue), cytoskeleton staining with Phalloidin (green).

**Fig. 3** PHK26 labeled exosomes engulfed by MAECs. Representative image of PHK-26 (red) and Phalloidin (green) double-immunostaining in MAECs. Nuclei were stained with DAPI (blue). Scale bar = 200 mm

**Expressions of STC-1 in ADSCs, their exosomes, and MAECs**

Representative Western blot pictures of STC-1 in ADSCs, their exosomes, and MAECs engulfing ADSC-Exo are shown in Fig. 4. Densitometric analysis revealed that compared with the untransfected groups, transfection of STC-1 markedly enhanced the expressions of STC-1 in ADSCs, their exosomes, and MAECs engulfing ADSC-Exo (*P* < .05).

**Fig. 4** Expressions of STC-1 in ADSCs, their exosomes, and MAECs engulfing ADSC-Exo. a b c Representative Western blot pictures showing STC-1 expression. d Densitometric quantification of STC-1 expression in S-ADSC group, vs untransfected. e Densitometric quantification of STC-1 expression in S-ADSC-Exo group, vs untransfected. f Densitometric quantification of STC-1 expression in S-Exo-MAEC group, vs untransfected. *P* < .05, compared with untransfected groups. S-ADSC, ADSCs transfected with STC-1; S-Exo-MAEC, MAEC engulfing S-ADSC-Exo.

**Reendothelialization effect *in vitro***

After activation of NLRP3 inflammasome, the scratch test was performed to assess the lateral migration capacity of MAECs. Compared with the control group and the ADSC-Exo group, excellent lateral migration capacity was observed in the S-ADSC-Exo group (Fig. 5a). Blank area calculation showed that the S-ADSC-Exo group had the fastest migration speed at time intervals (6 h, 12 h, 24 h) (Fig. 5c). Meanwhile, compared with the control group, recovery after scratching was faster in the ADSC-Exo group.

To assess the angiogenesis effect of S-ADSC-Exo on MAECs, tube forming experiment was performed. The results revealed that tube-forming ability of MAECs was significantly improved in the group incubated with S-ADSC-Exo. As shown in Fig. 5b, d, tube density in the S-ADSC-Exo group was highest compared with the control group and the ADSC-Exo group.

**Fig. 5** Reendothelialization effect *in vitro*. a Representative pictures showing lateral migration capacity at the time intervals (6h, 12h, and 24h); b Representative pictures showing tube forming; c Calculation of blank area according to picture A. *P* < .05, S-ADSC-Exo vs control. $P$ < .05, ADSC-Exo vs control. #P < .05,
S-ADSC-Exo vs ADSC-Exo; d Calculation of tube density according to picture B. \( *P < 0.05 \), vs control. \#P < 0.05, S-ADSC-Exo vs ADSC-Exo. Scale bar = 200 mm

**Mechanism of S-ADSC-Exo promoting reendothelialization in vitro**

Results of qRT-PCR are summarized in Fig. 6a. The expression of NLRP3 decreased slightly in the ADSC-Exo group (\( P = 1.000 \), vs control). Transfection of STC-1 significantly decreased the expression of NLRP3 in the S-ADSC-Exo group (\( P < 0.05 \), vs control). The expression of Caspase-1 decreased slightly in the ADSC-Exo group (\( P = 0.94 \), vs control). Transfection of STC-1 significantly decreased the expression of Caspase-1 in the S-ADSC-Exo group (\( P < 0.05 \), vs control). The expression of IL-1\( \beta \) decreased slightly in the ADSC-Exo group (\( P = 0.89 \), vs control). Transfection of STC-1 significantly decreased the expression of IL-1\( \beta \) in the S-ADSC-Exo group (\( P < 0.05 \), vs control).

Representative western blot pictures of NLRP3, Caspase-1, and IL-1\( \beta \) expressions were shown in Fig. 6b. Densitometric analysis revealed that transfection of STC-1 markedly decreased the expression of NLRP3 in the S-ADSC-Exo group (\( P < 0.05 \), vs control, Fig. 6c). There was no significant difference in the expression of NLRP3 between the control group and the ADSC-Exo group. Densitometric analysis revealed that transfection of STC-1 markedly decreased the expression of Caspase-1 in the S-ADSC-Exo group (\( P < 0.05 \), vs control, Fig. 6c). There was no significant difference in the expression of caspase-1 between the control group and the ADSC-Exo group. Densitometric analysis revealed that transfection of STC-1 markedly decreased the expression of IL-1\( \beta \) in the S-ADSC-Exo group (\( P < 0.05 \), vs control, Fig. 6c). There was no significant difference in the expression of IL-1\( \beta \) between the control group and the ADSC-Exo group.

**Fig. 6** Mechanism of S-ADSC-Exo promoting reendothelialization in vitro. a Expressions of NLRP3, caspase-1, and IL-1\( \beta \). b Representative western blot pictures showing expressions of NLRP3, Caspase-1, and IL-1\( \beta \). c Densitometric quantification of NLRP3, Caspase-1, and IL-1\( \beta \) expression. \( \star P < 0.05 \), vs control. EC, endothelial cell.

**Ros levels in carotid arteries**

Compared with the control group, levels of ROS in the ADSC-Exo and S-ADSC-Exo group were lower (Fig. 7). Compared with the ADSC-Exo group, level of ROS in the S-ADSC-Exo group was lower.

**Fig. 7** The level of ROS in carotid arteries after mechanical injury. \( \star P < 0.05 \), vs control. \#P < 0.05, S-ADSC-Exo vs ADSC-Exo.

**Mechanism of S-ADSC-Exo promoting reendothelialization in carotid artery tissue**

Representative western blot pictures of NLRP3, Caspase-1, and IL-1\( \beta \) were shown in Fig. 8a. Densitometric analysis revealed that transfection of STC-1 markedly decreased the expression of NLRP3 in the S-ADSC-Exo group (\( P < 0.05 \), vs control, Fig. 8b). There was no significant difference in the expression of NLRP3 between the control group and the ADSC-Exo group. Densitometric analysis revealed that...
transfection of STC-1 markedly decreased the expression of Caspase-1 in the S-ADSC-Exo group (P< .05, vs control, Fig. 8b). There was no significant difference in the expression of Caspase-1 between the control group and the ADSC-Exo group. Densitometric analysis revealed that transfection of STC-1 markedly decreased the expression of IL-1β in the S-ADSC-Exo group (P< .05, vs control, Fig. 8b). There was no significant difference in the expression of IL-1β between the control group and the ADSC-Exo group.

Fig. 8 Mechanism of S-ADSC-Exo promoting reendothelialization in vivo. a Representative western blot pictures showing expressions of NLRP3, Caspase-1, and IL-1β. b Densitometric quantification of NLRP3, caspase-1, and IL-1β expression. *P< .05, vs control.

Histological examination of the post-injury carotid artery

Representative sections of carotid endarterium stained with CD31 (red), NLRP3 inammasome (green), and merge (orange) are shown in Fig. 9a. As summarized in Figure 9b, d, the expressions of NLRP3, Caspase-1 and IL-1β in the S-ADSC-Exo group were much lesser than that in the control group and that in the ADSC-Exo group. HE staining (Fig. 9a, d) results showed that the thickness of arterial wall was much thinner in the S-ADSC-Exo group, compared with the control group and the ADSC-Exo group. Moreover, Even's blue staining demonstrated that the number of living endothelial cells was significantly greater than that in the control group and that in the ADSC-Exo group (Fig. 9e).

Fig. 9 Histological examinations of carotid arteries. a Representative images of IF staining, HE staining and Even's blue staining. b Quantification of NLRP3 expression. c Quantification of Caspase-1 expression. d Quantification of IL-1β expression. e Quantification of thickness of arterial wall. IF, immunofluorescence. HE, hematoxylin-eosin. *P< .05, vs control. Scale bar = 20 mm.

Discussion

The salient findings revealed by this current study are that the reendothelialization effect of S-ADSC-Exo on post-injury carotid artery is associated with enhanced lateral migration capacity of MAECs, promoting angiogenesis, decreased expression of NLRP3 inammasome in vitro and in vivo, suppressed negative remodeling of arterial wall.

ADSCs are abundant in source, easy to harvest and isolate, can robustly release exosomes, and play a crucial role in tissue repair [24]. Collective data have indicated that exosomes released from ADSCs conduct vascular repair by promoting vascular plasticity [25], enhancing angiogenesis [25], improving post-injury vascular regeneration [26], and regulating autophagy [27]. We revealed that exosomes from genetically modified ADSCs induced reendothelialization effects on post-injury carotid endarterium as evidenced by improvement of carotid artery remodeling and survival of endothelial cells. Inflammatory response is consequence of mechanical injury using endovascular techniques and may be a principal contributor to complex inflammatory reactions of endothelial cells [28]. NLRP3 inflammasome are a group of intracellular protein complexes produced during inflammation activation, and act as innate
immune signal receptor to initiate inflammatory response. IL-1β, as an important member and a major executor of NLRP3 inflammasome, is strongly expressed in the chronic inflammation and restenosis after endovascular treatment [11, 18]. In the current study, the elevation of NLRP3 inflammasome (NLRP3, Caspase-1, and IL-1β) after scratching was significantly inhibited by exosomes from ADSCs overexpressing STC-1, suggesting that exosomes may account for reendothelialization via anti-inflammation effects. It is worth noting that compared with the control group, the lateral migration capacity and the tube density is higher in the ADSC-Exo group. Although STC-1 was highlighted, some cargoes aside from STC-1 in ADSC-Exo may contribute to the reendothelialization and angiogenesis, which needs to be further investigated.

RNAs are important regulatory factors delivered by exosomes to target cells. Increasing reports indicates that ADSC-derived exosomes regulate target cell protein expression and cell morphological change. ADSC-derived exosomes promote wound-healing in diabetic mice through microRNA-128-3p/SIRT1 mechanism [27]. Via exosomes, ADSCs communicate with cardiomyocytes and macrophages to ameliorate ischemic injury by activating S1P/SK1/S1PR1 signaling pathway and foster M2 polarization [29]. Microglia-induced neural inflammatory injury can be suppressed by ADSC-exosomes through the NF-kB/MAPK pathway [30]. All these data show that the function ADSC-exosome can be enhanced by changing their RNA content. STC-1 is a conserved glycoprotein that can directly act on mitochondria, regulate oxidative phosphorylation and inhibit inflammatory reactions [22, 31]. By inhibiting oxidative stress, STC-1restrain renal ischemia-reperfusion injury through ROS-mediated multiple signaling pathways [32]. Through being encapsulated within the exosomes, the STC-1 or other RNAs is protected from the degradation of protease or RNase [33]. Compared with untransfected groups, transfection of STC-1 enhanced the levels of STC-1 in ADSCs, released exosomes, and MAECs engulfing exosomes. The content of ROS was decreased in carotid arteries after in vivo administration of S-ADSC-Exo. However, the same result occurred in the ADSC-Exo group, there may be some other active biomolecules within the exosomes played the alike role of STC-1, which needs to be further studied.

To elucidate the potential reendothelialization effect of STC-1 on post-injury carotid endarterium, possibilities for the related proteins were explored. STC-1 is indicated to be an endogenous regulator of ROS [34], and ROS is the key signal to activate NLRP3 inflammasome [35]. ROS is a major mediator of the inflammation follows mechanical injury of endovascular treatment. Mechanical injury triggers an overproduction of ROS, which leads to complex change of intracellular protein expressions and results in cell apoptosis and pyroptosis [36]. ROS can activate NLRP3 inflammasome by promoting the link of Thioredoxin-interacting protein (TXNIP) and NLRP3 [37]. The expressions of NLRP3, Caspase-1, and IL-1β were detected to be inhibited in MAECs and in carotid arteries, indicating that the expressions of NLRP3 inflammasome were in consistent with the content of ROS and ROS was involved in the activation of NLRP3 inflammasome. Another important finding in this study was that STC-1 was transferred into carotid arteries by STC-1 enriched ADSC-exosomes, and the expressions of NLRP3 inflammasome were decreased as evidenced by immunofluorescence staining. In the meanwhile, the negative remodeling of arterial wall and the survival of endothelial cells were promoted. Therefore, it is plausible that STC-1 is mediator of NLRP3 inflammasome by which STC-1 enriched ADSC-exosomes attenuated the oxidative
stress after mechanical injury of carotid artery and mediated reendothelialization effects on post-injury carotid endarterium compared with ADSC-derived exosomes.

Conclusions

Exosomes derived from genetically modified ADSCs conduct reendothelialization effects on post-injury carotid endarterium through STC-1/NLRP3 inflammasome pathway.

Abbreviations

STC-1: Stanniocalcin-1; ADSC: adipose-derived mesenchymal stem cells; MAEC: mice arterial endothelial cells; MSC: mesenchymal stem cells; ROS: reactive oxygen species; S-ADSC-Exo: STC-1-ADSC-Exosome; IL: interleukin; PTA: percutaneous transluminal angioplasty; TXNIP: thioredoxin-interacting protein; IF: immunofluorescence; HE: hematoxylin-eosin; EC: endothelial cell; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Declarations

Acknowledgements

None

Author contribution

Kun Liu, Huihua Shi, and Zhiyou Peng contributed equally to this work. Xiaoyu Wu, Xinwu Lu and Weimin Li contributed equally to this work. Kun Liu, Huihua Shi and Zhiyou Peng contributed to experimental operation, data collection and analysis, manuscript writing. Xiaoyu Wu and Xinwu Lu contributed to model development, code development, and editing the manuscript. Weimin Li contributed to manuscript proofreading.

Funding

This work was supported by the National Natural Science Foundation of China (Grant 81701801, 81971712, 81701842, and 81870346).

Availability of data and materials

The data and materials support that findings could be found.

Ethics approval and consent to participate

In this study, all animal experiments were approved by the Committee on the Use and Care on Animals (Shanghai Jiao Tong University School of Medicine, Shanghai, China) and performed in accordance with institution guidelines.
Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author details

1Department of Vascular Surgery, Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, China.

References

1. Zhu Z, Yu W. Update in the treatment of extracranial atherosclerotic disease for stroke prevention. Stroke Vasc Neurol. 2020;5(1):65–70.
2. Saw J. Carotid artery stenting for stroke prevention. Can J Cardiol. 2014;30(1):22–34.
3. Wangqin R, Krafft PR, Piper K, Kumar J, Xu K, Mokin M, Ren Z. Management of De Novo Carotid Stenosis and Postintervention Restenosis-Carotid Endarterectomy Versus Carotid Artery Stenting-a Review of Literature. Transl Stroke Res. 2019;10(5):460–74.
4. Chaabane C, Otsuka F, Virmani R, Bochaton-Piallat ML. Biological responses in stented arteries. Cardiovasc Res. 2013;99(2):353–63.
5. Wu B, Mottola G, Schaller M, Upchurch GR Jr, Conte MS. Resolution of vascular injury: Specialized lipid mediators and their evolving therapeutic implications. Mol Aspects Med. 2017;58:72–82.
6. Wang Y, Liu J, Ma J, Sun T, Zhou Q, Wang W, Wang G, Wu P, Wang H, Jiang L, et al. Exosomal circRNAs: biogenesis, effect and application in human diseases. Mol Cancer. 2019;18(1):116.
7. Piffoux M, Nicolas-Boluda A, Mulens-Arias V, Richard S, Rahmi G, Gazeau F, Wilhelm C, Silva AKA. Extracellular vesicles for personalized medicine: The input of physically triggered production, loading and theranostic properties. Adv Drug Deliv Rev. 2019;138:247–58.
8. Bouchareychas L, Duong P, Covarrubias S, Alsop E, Phu TA, Chung A, Gomes M, Wong D, Meechoovet B, Capili A, et al. Macrophage Exosomes Resolve Atherosclerosis by Regulating Hematopoiesis and Inflammation via MicroRNA Cargo. Cell Rep. 2020;32(2):107881.
9. Loyer X, Zlatanova I, Devue C, Yin M, Howangyin KY, Klahimmon P, Guerin CL, Kheloufi M, Vilar J, Zannis K, et al. Intra-Cardiac Release of Extracellular Vesicles Shapes Inflammation Following Myocardial Infarction. Circ Res. 2018;123(1):100–6.
10. Ma T, Sun J, Zhao Z, Lei W, Chen Y, Wang X, Yang J, Shen Z. A brief review: adipose-derived stem cells and their therapeutic potential in cardiovascular diseases. Stem Cell Res Ther. 2017;8(1):124.
11. Cai C, Kilari S, Zhao C, Simeon ML, Misra A, Li Y, van Wijnen AJ, Mukhopadhyay D, Misra S. Therapeutic Effect of Adipose Derived Mesenchymal Stem Cell Transplantation in Reducing
Restenosis in a Murine Angioplasty Model. J Am Soc Nephrol. 2020.

12. Lin Y, Zhu W, Chen X. The involving progress of MSCs based therapy in atherosclerosis. Stem Cell Res Ther. 2020;11(1):216.

13. Munoz MF, Arguelles S, Guzman-Chozas M, Guillen-Sanz R, Franco JM, Pintor-Toro JA, Cano M, Ayala A. Cell tracking, survival, and differentiation capacity of adipose-derived stem cells after engraftment in rat tissue. J Cell Physiol. 2018;233(10):6317–28.

14. Yan W, Jiang S. Immune Cell-Derived Exosomes in the Cancer-Immunity Cycle. Trends Cancer. 2020;6(6):506–17.

15. Ko KW, Yoo YI, Kim JY, Choi B, Park SB, Park W, Rhim WK, Han DK. Attenuation of Tumor Necrosis Factor-alpha Induced Inflammation by Umbilical Cord-Mesenchymal Stem Cell Derived Exosome-Mimetic Nanovesicles in Endothelial Cells. Tissue Eng Regen Med. 2020;17(2):155–63.

16. Fan B, Li C, Szalad A, Wang L, Pan W, Zhang R, Chopp M, Zhang ZG, Liu XS. Mesenchymal stromal cell-derived exosomes ameliorate peripheral neuropathy in a mouse model of diabetes. Diabetologia. 2020;63(2):431–43.

17. Ye Y, Zhang X, Xie F, Xu B, Xie P, Yang T, Shi Q, Zhang CY, Zhang Y, Chen J, et al. An engineered exosome for delivering sgRNA:Cas9 ribonucleoprotein complex and genome editing in recipient cells. Biomater Sci. 2020;8(10):2966–76.

18. Wei W, Li XX, Xu M. Inhibition of vascular neointima hyperplasia by FGF21 associated with FGFR1/Syk/NLRP3 inflammasome pathway in diabetic mice. Atherosclerosis. 2019;289:132–42.

19. Swanson KV, Deng M, Ting JP. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat Rev Immunol. 2019;19(8):477–89.

20. Sorbara MT, Girardin SE. Mitochondrial ROS fuel the inflammasome. Cell Res. 2011;21(4):558–60.

21. Li X, Zhou H, Guo D, Hu Y, Fang X, Chen Y, Zhang F. Oxidative stress and inflammation: Early predictive indicators of multiple recurrent coronary in-stent chronic total occlusions in elderly patients after coronary stenting. IUBMB Life. 2020;72(5):1023–33.

22. Oh JY, Ko JH, Lee HJ, Yu JM, Choi H, Kim MK, Wee WR, Prockop DJ. Mesenchymal stem/stromal cells inhibit the NLRP3 inflammasome by decreasing mitochondrial reactive oxygen species. Stem Cells. 2014;32(6):1553–63.

23. Wang Y, Chu Y, Li K, Zhang G, Guo Z, Wu X, Qiu C, Li Y, Wan X, Sui J, et al. Exosomes Secreted by Adipose-Derived Mesenchymal Stem Cells Foster Metastasis and Osteosarcoma Proliferation by Increasing COLGALT2 Expression. Front Cell Dev Biol. 2020;8:353.

24. Cai J, Wu J, Wang J, Li Y, Hu X, Luo S, Xiang D. Extracellular vesicles derived from different sources of mesenchymal stem cells: therapeutic effects and translational potential. Cell Biosci. 2020;10:69.

25. Hoang DH, Nguyen TD, Nguyen HP, Nguyen XH, Do PTX, Dang VD, Dam PTM, Bui HTH, Trinh MQ, Vu DM, et al. Differential Wound Healing Capacity of Mesenchymal Stem Cell-Derived Exosomes Originated From Bone Marrow, Adipose Tissue and Umbilical Cord Under Serum- and Xeno-Free Condition. Front Mol Biosci. 2020;7:119.
26. Li X, Xie X, Lian W, Shi R, Han S, Zhang H, Lu L, Li M. Exosomes from adipose-derived stem cells overexpressing Nrf2 accelerate cutaneous wound healing by promoting vascularization in a diabetic foot ulcer rat model. Exp Mol Med. 2018;50(4):29.

27. Shi R, Jin Y, Hu W, Lian W, Cao C, Han S, Zhao S, Yuan H, Yang X, Shi J, et al. Exosomes derived from mmu_circ_0000250-modified adipose-derived mesenchymal stem cells promote wound healing in diabetic mice by inducing miR-128-3p/SIRT1-mediated autophagy. Am J Physiol Cell Physiol. 2020;318(5):C848–56.

28. Davis C, Fischer J, Ley K, Sarembock IJ. The role of inflammation in vascular injury and repair. J Thromb Haemost. 2003;1(8):1699–709.

29. Deng S, Zhou X, Ge Z, Song Y, Wang H, Liu X, Zhang D. Exosomes from adipose-derived mesenchymal stem cells ameliorate cardiac damage after myocardial infarction by activating S1P/SK1/S1PR1 signaling and promoting macrophage M2 polarization. Int J Biochem Cell Biol. 2019;114:105564.

30. Feng N, Jia Y, Huang X. Exosomes from adipose-derived stem cells alleviate neural injury caused by microglia activation via suppressing NF-kB and MAPK pathway. J Neuroimmunol. 2019;334:576996.

31. Sheikh-Hamad D. Mammalian stanniocalcin-1 activates mitochondrial antioxidant pathways: new paradigms for regulation of macrophages and endothelium. Am J Physiol Renal Physiol. 2010;298(2):F248-54.

32. Liu D, Shang H, Liu Y. Stanniocalcin-1 Protects a Mouse Model from Renal Ischemia-Reperfusion Injury by Affecting ROS-Mediated Multiple Signaling Pathways. Int J Mol Sci. 2016;17(7).

33. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654–9.

34. Yang K, Yang Y, Qi C, Ju H. Effects of porcine STC-1 on cell metabolism and mitochondrial function. Gen Comp Endocrinol. 2020;286:113298.

35. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat Rev Immunol. 2010;10(3):210–5.

36. Robinson N, Ganesan R, Hegedus C, Kovacs K, Kufer TA, Virag L. Programmed necrotic cell death of macrophages: Focus on pyroptosis, necroptosis, and parthanatos. Redox Biol. 2019;26:101239.

37. Choe JY, Kim SK. Quercetin and Ascorbic Acid Suppress Fructose-Induced NLRP3 Inflammasome Activation by Blocking Intracellular Shuttling of TXNIP in Human Macrophage Cell Lines. Inflammation. 2017;40(3):980–94.