Small extracellular vesicles from rat plasma promote migration and proliferation of vascular smooth muscle cells

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ABSTRACT. Small extracellular vesicles (sEV) contain various molecules and mediate cell-to-cell communication under both physiological and pathological conditions. We have recently reported that sEV isolated from plasma of normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) regulate systemic blood pressure. The initiation and development of hypertension partly rely on proliferation and migration of vascular smooth muscle cells (SMCs) followed by the structural remodeling of vascular wall. In the present study, we examined the effects of plasma sEV in WKY and SHR on the proliferative and migratory functions of primary rat aortic SMCs. There was no difference in the concentration and size distribution of plasma sEV between WKY and SHR, while the protein expression of CD81 in plasma sEV from SHR was lower than that from WKY. Both plasma sEV from WKY and SHR were internalized into SMCs and stimulated the migration and proliferation with a similar potency. In summary, we, for the first time, demonstrated that plasma sEV in WKY and SHR are physiologically active in terms of proliferative and migratory functions, however, these effects do not seem to be related to the pathogenesis of hypertension development.

KEY WORDS: hypertension, plasma, small extracellular vesicles, spontaneously hypertensive rat, vascular smooth muscle cell
MATERIALS AND METHODS

Animals

All animal care and procedures were conducted in conformity with the institutional guideline of School of Veterinary Medicine, the Kitasato University. The animal study was approved by the ethical committee of School of Veterinary Medicine, the Kitasato University. Male Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR, Hoshino Laboratory Animals, Inc., Ibaraki, Japan), and normal Wistar rats (CLEA Japan, Inc., Tokyo, Japan) were introduced. The rats can freely take food (CE2, CLEA Japan) and tap water.

sEV isolation from plasma

sEV were isolated from plasma of WKY (WsEV) and SHR (SsEV) by the precipitation with polyethylene-glycol and ultracentrifugation [27]. WKY and SHR (6-week-old) were deeply anesthetized with urethane (Sigma-Aldrich, St. Louis, MO, USA) (1.5 g/kg, i.p.), and blood was drawn via an inferior vena cava. The collected blood samples were gently mixed with heparin at a final concentration of 1 U/ml and centrifuged (1,000 × g, room temperature: RT, 10 min) by using a microcentrifuge (model 3740, Kubota Corp., Tokyo, Japan) to separate plasma. The plasma samples were centrifuged (10,000 × g, 4°C, 10 min), and the supernatant was mixed with an equal volume of sterilized polyethylene-glycol solution consisting of 16% polyethylene-glycol (MW=6,000, FUJIFILM Wako Pure Chemical, Osaka, Japan) and 1 M NaCl (Nacalai Tesque, Kyoto, Japan) by inverting (4°C, overnight). After centrifugation (2,500 × g, 4°C, 15 min), the supernatants were discarded. The pellets were resuspended in sterilized phosphate-buffered saline (PBS) by vigorous vortex (RT, 30 min) and ultracentrifuged (164,071 × g, 4°C, 35 min) by using an Optima XL-80K ultracentrifuge with a swing rotor SW 55 Ti (Beckman Coulter Inc., Miami, FL, USA). The pellets were resuspended in sterilized PBS, which were used for the following examinations.

Concentration and size distribution of plasma sEV

The concentration and size distribution of the isolated vesicles were measured by a tunable resistive pulse sensing (TRPS) method using a qNANO instruments with an NP150 nanopore at 46.0–47.0 mm stretch (IZON Science, Christchurch, New Zealand), which was coated with a TRPS Reagent Kit (IZON Science) for the prevention of sEV adhesion according to a manufacturer’s instruction [27]. Raw data were standardized by carboxylated-polystyrene particles with a known diameter of 110 nm.

Protein expression in plasma sEV

The protein expression in plasma sEV was determined by Western blotting as previously described [27]. Protein lysates of sEV were extracted using a radio-immunoprecipitation assay buffer containing 20 mM Tris-HCl (pH7.5), 150 mM NaCl, 10 mM MgCl2, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% protease inhibitor mixture, and 1% phosphatase inhibitor mixture (Nacalai Tesque) (4°C, 10 min). Protein concentration was measured by using a Pierce BCA protein assay kit (Pierce, Rockford, IL, USA). After equal amounts of protein were separated by SDS-PAGE (7.5 and 14%, 80–120 V, 1.5 hr), they were transferred to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, USA) (400 mA, 1.5 hr). To confirm the equal loading of protein, the membranes were stained with 0.1% Ponceau-S/5% acetic acid solution. The Ponceau-S stained membranes were scanned in visible light by using the ATTO light capture system (ATTO, Tokyo, Japan). The total density of all the visible bands in each lane was measured as the amount of total protein using CS analyzer 3.0 software (ATTO). After being digested with an M199 medium containing collagenase again (37°C, 30 min). Then, the rings were minced by pipetting and centrifuged (200 × g, 5 min, 4°C). The supernatant was discarded, and the pellet was resuspended in 2.5 g/ml trypsin/1 mM EDTA solution (Nacalai Tesque) diluted 2 times by TBS. After incubation (37°C, 30 min) and dispersion by pipetting, they were collected in a new tube and treated with a trypsin neutralizer solution (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was discarded after centrifugation (200 × g, 5 min, 4°C), and the cells were resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM, FUJIFILM Wako Pure Chemical) supplemented with 20% fetal bovine serum (FBS). The resuspended cells were seed in a 35 mm-culture dish and passaged at
confluent. They (passage 4–10) were cultured in DMEM supplemented with 10% FBS and used for the following experiments.

**Cell migration analysis by a boyden chamber assay**

We examined the migration of RASM by a boyden chamber assay [36, 43]. Briefly, the polycarbonate membranes with an 8 µm pore size (Costar, Cambridge, MA, USA) were coated by 2% gelatin (FUJIFILM Wako Pure Chemical) in ddH₂O (37°C, 30 min). RASM (5.0 × 10⁶ cells/well) were seeded in the upper chamber and treated for 48 hr with WsEV, SsEV (0.1, 0.3, 1.0 × 10⁸ particles/mℓ) or PBS (Cont). After the treatment, the membranes were fixed with 100% methanol (RT, 15 min) and stained with a Giemsa stain solution (Nacalai Tesque) diluted 15 times by ddH₂O (RT, 20 min). Non-migrated cells in the upper side of the polycarbonate membrane were wiped with a cotton swab. The migrated cells in the lower side were photographed and randomly counted on the basis of those of nuclei in three fields (>100) by using a light microscope (CKX31, Olympus Corp., Tokyo, Japan) equipped with a CCD camera (True Chrome II Plus, TUCSEN, Fujian, China).

**Cell proliferation analysis by a bromodeoxyuridine (BrdU) incorporation assay**

We examined the proliferation of RASM with a BrdU incorporation assay kit (Exalpha Biologicals, Shirley, MA, USA) as described previously [36, 43]. Briefly, RASM (4.0 × 10⁶ cells/well) were seeded on a 96-well culture plate and treated for 48 hr with WsEV, SsEV (0.1, 0.3, 1.0 × 10⁸ particles/mℓ) or PBS (Cont). The BrdU reagent was added to the wells for 36 hr in the presence of sEV or PBS. After the treatment, the cells were fixed with a fixing solution (RT, 30 min) and washed with the wash buffer three times. Then, they were treated with an anti-BrdU antibody (RT, 1 hr) and incubated with an HRP-conjugated anti-mouse IgG (1:2,000 dilution, RT, 30 min). Tetra-methyl benzidine (TMB) peroxidase substrate was added (RT, 30 min) followed by the addition of the stop solution (Tristart LB941, Berthold Technologies, Bad Wildbad, Germany).

**Uptake of sEV labeled with PKH67**

To assess the uptake of plasma sEV into RASM, they were treated with WsEV or SsEV, which were labeled with PKH67 green fluorescence dye (Sigma Aldrich). WsEV or SsEV were reacted with PKH67 (4 µM, RT, 3 min), which was stopped with the same volume of sterilized PBS containing 10% bovine serum albumin (Nacalai Tesque). To wash the excess dye, the sEV-PKH67 solution was taken into a tube containing a sterilized 0.971 M sucrose solution in the bottom and ultracentrifuged (164,071 × g, 4°C, 35 min). The supernatants were removed by pipetting and the pellets were resuspended in sterilized PBS. RASM were treated for 2 hr with PKH67-labeled sEV (1.0 × 10⁸ particles/mℓ) or PKH67-reacted PBS (Cont) and fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical) (4°C, 10 min). Nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI, Dojindo stain solution (Nacalai Tesque) diluted 15 times by ddH₂O (RT, 20 min) and photographed by using a fluorescence microscope (BX-51) with a digital camera (FUJIFILM Wako Pure Chemical) (4°C, 30 min). The fluorescence dye (Sigma Aldrich). WsEV or SsEV were reacted with PKH67 (4 µM, RT, 3 min), which was stopped with the same volume of sterilized PBS containing 10% bovine serum albumin (Nacalai Tesque). To wash the excess dye, the sEV-PKH67 solution was taken into a tube containing a sterilized 0.971 M sucrose solution in the bottom and ultracentrifuged (164,071 × g, 4°C, 35 min). The supernatants were removed by pipetting and the pellets were resuspended in sterilized PBS. RASM were treated for 2 hr with PKH67-labeled sEV (1.0 × 10⁸ particles/mℓ) or PKH67-reacted PBS (Cont) and fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical) (4°C, 10 min). Nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI, Dojindo Laboratories, Kumamoto, Japan) (RT, 10 min) and photographed by using a fluorescence microscope (BX-51) with a digital camera (DP74) and CellSens standard dimension ver. 1.18 software (Olympus). Fluorescence density in a single cell was measured using an ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis**

Data are shown as means ± standard error of mean (SEM). Statistical analyses were performed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons and by Student’s t-test between two groups. Values of P<0.05 were considered statistically significant.

**RESULTS**

**Concentration and size distribution of plasma sEV from WKY and SHR**

We first verified whether the vesicles isolated from plasma of WKY and SHR by the precipitation with polyethylene-glycol and ultracentrifugation exhibit the expected size profiles of sEV as previously reported [40]. The isolated vesicles from plasma of WKY and SHR were distributed within the expected ranges with a diameter of 100–150 nm as determined by the tunable resistive pulse sensing analyses (Fig. 1a, b, n=3). These results suggest the successful plasma sEV isolation from WKY and SHR. Next, we compared the concentration and size between plasma sEV from WKY (WsEV) and SHR (SsEV). There was no significant difference in total concentration (Fig. 1c, n=3, 1.31 ± 0.07 × 10¹³ particles/mℓ) between WsEV and SsEV.

There was no difference in the expression of CD63 between WsEV and SsEV (Fig. 2a, n=3), while the expression of CD81 in SsEV was significantly lower than that in WsEV (Fig. 2b, n=3, P<0.05). The removal of other types of EVs and contaminating proteins was also examined. In WsEV or SsEV, the protein expression of large EV marker (CD9) and the plasma contaminants (albumin) were significantly lower than those in the positive control (Fig. 2c, n=3, P<0.01 vs. Large EV; Fig. 2d, n=3, P<0.01 vs. Plasma).

**Effects of WsEV and SsEV on migration and proliferation in vascular SMCs**

Next, we examined the effects of WsEV and SsEV on the cellular functions in RASM. WsEV (0.3, 1.0 × 10⁸ particles/mℓ, 48 hr)
and SsEV (1.0 × 10^8 particles/ml, 48 hr) significantly increased the migration in RASM (Fig. 3a, n=3, 0.3 × 10^8 particles/ml WsEV, P<0.05 vs. Cont; 1.0 × 10^8 particles/ml WsEV, P<0.01 vs. Cont). Furthermore, WsEV (1.0 × 10^8 particles/ml, 48 hr) and SsEV (1.0 × 10^8 particles/ml, 48 hr) significantly increased the proliferation in RASM (Fig. 3b, n=6, P<0.01 vs. Cont). There was no difference in the migratory and proliferative effects between WsEV and SsEV.

**DISCUSSION**

The major findings of the present study are as follows: 1) There was no significant difference in the concentration and size distribution between WsEV and SsEV, while the protein expression of CD81 differed. 2) Both WsEV and SsEV were internalized into RASM, and then stimulated the migration and proliferation with a similar potency.

In the present study, the concentration of plasma sEV isolated from WKY and SHR was approximately 1.0–1.5 × 10^10 particles/ml (Fig. 1). No other groups so far have reported the concentration of plasma sEV from WKY and SHR, while the concentration of plasma sEV in Sprague-Dawley [22, 35, 37, 46], Wistar [6], and Fisher 344 [9] rats was reported to be ranging between 10^8–10^12 particles/ml. Therefore, the concentration of our sEV would be within the reasonable ranges and also was enough for the following analyses. In the present study, RASM were treated with sEV at 1.0 × 10^7–10^8 particles/ml (Figs. 3, 4). Other studies examined the effects of EV on cultured cells at wide-ranging concentrations between 10^5–10^9 particles/ml [5, 12, 50]. Then, there was no obvious deviation in the concentrations of sEV used between ours and others'.

A variety of proteins, such as tetraspanin (CD9, CD63, and CD81), major histocompatibility complex, and integrin, are expressed on the surface of sEV [39, 45]. In the present study, the protein expression of CD81 in SsEV was lower than WsEV (Fig. 2). It is assumed that the differences in the origins of SsEV and WsEV exist, since the expression profiles of surface proteins on sEV are different dependent on the EV-secreting cell types [24, 35]. Moreover, the surface proteins could potentially affect the
amounts of internalized sEV into the cell. For example, a degradation of surface protein on sEV by proteinase K can suppress the uptake of sEV in human ovarian cancer cells [15]. In addition, the anti-CD9 or -CD81 antibody prevents the HIV-1 entry into human T-lymphoblastoid reinforced by sEV [34]. In the present study, however, there was no significant difference in the amounts of uptake between WsEV and SsEV (Fig. 4). Therefore, CD81 would have no crucial role in the uptake of sEV. In addition, CD81 mediates antigen presentation [2], and the upregulation of CD81 induced by oxidative stress in endothelial cells was reported to promote the monocyte adhesion [31]. Although the increased concentration of sEV and the associated upregulation of CD81 in plasma of type II diabetes model rats were reported [14], downregulation of CD81 in sEV under pathological condition has not been reported to the best of our knowledge. Then the meanings of the decreased CD81 expression in SsEV remain to be clarified, which needs to be extensively explored in the future study.

Both WsEV and SsEV similarly stimulated the migration and proliferation of RASM in the present study (Fig. 3). The sEV derived from fetal bovine serum (FBS) and plasma of Sprague-Dawley rats were also reported to stimulate cell migration [33, 37]. These results suggest that the circulating sEV can activate cell migration or proliferation irrespective of their origins and that the observed effects of WsEV and SsEV on RASM are physiological but not pathological ones. We have recently reported that SsEV can mediate the increase of systolic blood pressure in WKY, while WsEV can mediate the decrease of systolic blood pressure in SHR [28]. Then, it is suggested that WsEV and SsEV would regulate the blood pressure via the actions on other tissues than vascular SMCs, including central nervous system, heart, kidney, and vascular endothelium, which potentially contribute to regulate the development of systemic hypertension [8, 23, 29, 32].

In summary, we, for the first time, demonstrated that plasma sEV in WKY and SHR are similarly uptaken into the cells and stimulate the migration and proliferation. Further researches are required to elucidate the underlying mechanisms of plasma sEV regulating pathology of systemic hypertension.
Fig. 3. Effects of small extracellular vesicles (sEV) in plasma of Wistar Kyoto rats (WKY) (WsEV) and spontaneously hypertensive rats (SHR) (SsEV) on migration and proliferation in vascular smooth muscle cells. (a) Migration of primary rat aortic smooth muscle cells (RASM) was determined by a boyden chamber assay. RASM were treated for 48 hr with WsEV, SsEV (0.1, 0.3 or 1.0 × 10^8 particles/ml) or PBS (Cont). We observed the migrated RASM which were stained with Giemsa using a phase-contrast microscope. Data were shown as fold increase relative to the migrated cell number in Cont. Results were expressed as means ± standard error of the mean (SEM) (n=3) in bar graph. Scale bar: 100 µm. *P<0.05, **P<0.01 vs. Cont. (b) Proliferation of RASM was determined by a bromodeoxyuridine (BrdU) incorporation assay. RASM were stimulated for 48 hr with WsEV, SsEV (0.1, 0.3 or 1.0 × 10^8 particles/ml) or PBS (Cont). In the presence of sEV or PBS, RASM were treated with BrdU for 36 hr. Incorporation of BrdU was measured by an immunostaining with anti-BrdU antibody. Data were shown as fold increase relative to Cont. Results were expressed as means ± SEM (n=6) in bar graph. **P<0.01 vs. Cont.

Fig. 4. Uptake of small extracellular vesicles (sEV) into cells. Primary rat aortic smooth muscle cells (RASM) were treated with plasma sEV of Wistar Kyoto rats (WKY) (WsEV) and spontaneously hypertensive rats (SHR) (SsEV) (1.0 × 10^8 particles/ml, 2 hr) which were labeled with PKH67 green fluorescence dye (4 µM). For negative control (Cont), RASM were treated with vehicle (PBS) reacted with PKH67 by the same procedure as WsEV and SsEV. After the cells were fixed with 4% paraformaldehyde, the nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI), which were observed by a fluorescence microscope. The green fluorescence density (intensity × area) in the cells relative to WsEV was shown as means ± standard error of the mean (n=3) in bar graph. Green: PKH67; Blue: DAPI. Scale bar: 100 µm.
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