Published in final edited form as:

J Control Release. 2016 February 28; 224: 208–216. doi:10.1016/j.jconrel.2016.01.024.

Cell Membrane-formed Nanovesicles for Disease-Targeted Delivery

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Abstract

Vascular inflammation is underlying components of most diseases. To target inflamed vasculature, nanoparticles are commonly engineered by conjugating antibody to the nanoparticle surface, but this bottom-up approach could affect nanoparticle targeting and therapeutic efficacy in complex, physiologically related systems. During vascular inflammation endothelium via the NF- κ B pathway instantly upregulates intercellular adhesion molecule 1 (ICAM-1) which binds integrin β_2 on neutrophil membrane. Inspired by this interaction, we created a nanovesicle-based drug delivery system using nitrogen cavitation which rapidly disrupts activated neutrophils to make cell membrane nanovesicles. Studies using intravital microscopy of live mouse cremaster venules showed that these vesicles can selectively bind inflamed vasculature because they possess intact targeting molecules of integrin β_2 . Administering of nanovesicles loaded with TPCA-1 (a NF- κ B inhibitor) markedly mitigated mouse acute lung inflammation. Our studies reveal a new top-down strategy for directly employing a diseased tissue to produce biofunctional nanovesicle-based drug delivery systems potentially applied to treat various diseases.

1. Introduction

The vascular endothelium lining the lumen of blood vessels regulates a variety of functions (1) including expression of proteins and release of plasma growth factors, and regulation of tissue fluid homeostasis via endothelial tight junction molecules. A monolayer of endothelium selectively transports plasma molecules and nanoparticles across vessel walls through transcytosis (2–4). Moreover, the vascular endothelium plays a central role in immunity to respond infection and tissue damage (5–7). Because endothelium essentially governs the systemic physiology, dysfunctional endothelium is underlying components of most diseases, for example cancer (8), atherosclerosis (9), sepsis (10), autoimmune disease

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Supporting Data: Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper, references unique to the sections appear only in the online paper. It includes Figure S1–12

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(5) and acute lung inflammation/injury (11), thus it is a pressing need to develop novel drug delivery platforms which selectively target diseased vasculature in reversing the progression of vascular disorders.

Vascular inflammation is a feature of immune response that is a movement of immune cells from one location to another, and the mechanism underlying the migration is regulated by intercellular adhesion molecules (12). At inflammation sites, endothelium rapidly upregulates intercellular adhesion molecule 1 (ICAM-1) mainly through the NF- κ B pathway to recruit leukocytes (13). Neutrophils, a type of polymorphonuclear leukocytes and the most abundant circulating leukocytes in human, are a central player in acute inflammation induced by infection or tissue damage (14). They are the first to migrate to inflammatory locations and are capable of eliminating pathogens, but dysregulated neutrophil recruitment and excessive vascular inflammation could cause organ failure and damage (14), such as acute lung inflammation and injury (11, 15).

To target inflamed vasculature, nanoparticles were commonly engineered by conjugating anti-ICAM-1 or peptides to the surface of nanoparticles (16, 17), however, the conjugation could impair their specificity and affinity to the target, in particular when the nanoparticles are administrated *in vivo* (18, 19). When inflammation occurs, neutrophils highly express integrin β_2 , which binds to endothelial cells via ICAM-1 molecules (14). Here we proposed a top-down strategy that uses an activated neutrophil as a building block to generate nanovesicles derived from neutrophil membrane and using these nanovesicles, we can selectively deliver drugs to inflamed vasculature in reversing acute lung inflammation and injury.

2. Materials and Methods

2.1 Reagents and Chemicals

LPS (*Escherichia coli* 0111:B4), formaldehyde solution and dimethyl sulfoxide (DMSO, purity >99.5%) were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human and mouse TNF- α (carrier-free, purity >98%), Alexa Fluor@647 anti-mouse CD31 antibody, Alexa Fluor@488 anti-mouse Gr-1 (Ly-6G/Ly-6C) antibody and ELISA kits for TNF- α and IL-6 were purchased from Biolegend Inc. (San Diego, CA). Human HL 60 cell lines was obtained from ATCC (Manassas, VA) and erythrocytes were purchased from Zen-Bio (Research Triangle Park, NC). Anti-ICAM-1 antibody and anti-integrin- β_2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TPCA-1 was purchased from Tocris Bioscience (Minneapolis, MN). Human vascular endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD). Formavar carbon film on 100 mesh nickel grid for TEM was obtained from Electron Microscopy Sciences. Diff-Quick dye was purchased from Polysciences Inc. (Warrington, PA). DiO (3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl]-, perchlorate) [Ex(484nm)\Em(501nm)] and DiI (3H-Indolium, 2-(3-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-ylidene)-1-propenyl)-3,3-dimethyl-1-o ctadecyl-, perchlorate) [(Ex(549nm)\Em(565nm)], DiD (3H-Indolium, 2-(5-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-ylidene)-1,3-pentadienyl)-3,3-dimethyl-1-octadecyl-, perchlorate) [Ex(640nm)\Em(670nm)], DAPI, Penicillin streptomycin (pen strep) and glutamine (100x) were purchased from Life

Technologies (Grand Island, NY). QuantiFluor dsDNA detection kit was purchased from Promega. PierceTM BCA protein assay kit was purchased from Thermo Fisher Scientific.

2.2 HL 60 cell culture and their activation

Human HL 60 cells were culture in RPMI1640 (Lonza, Walkersville, MD) supplemented with 10% (v/v) FBS and 1% (v/v) pen strep/glutamine. To activate HL 60 cells to express integrin β_2 , 1.3% (v/v.) DMSO was added into the culture medium and the cells were cultured for 4–6 days. Integrin β_2 expression was determined by Western blot.

2.3 Preparation of cell membrane-formed nanovesicles, their drug loading and fluorescent labeling

DMSO-treated HL 60 cells were harvested and washed with HBSS (without Ca^{2+} , Mg^{2+} and phenol red, Corning, Inc., Corning, NY). The cells were re-suspended in HBSS at a concentration of $1\text{--}5 \times 10^7/\text{ml}$. A 10–20 ml of suspension with 2.5 mM MgCl_2 was placed in a nitrogen cavitation vessel (Parr instrument, Moline, IL) under a pressure of 350–400 psi for 20 min and the pressure was quickly released to disrupt cells. To completely disrupt cells, the nitrogen cavitation was repeated twice. The resulting suspension was added with 2.5 mM EDTA and was centrifuged at 2,000 g at 4 °C for 30 min. The resulting supernatant was centrifuged at 100, 000 g at 4°C for 30 min (Ultra TLX Beckman). After the supernatant was removed, a pellet was suspended in 2 ml of HBSS. The suspension at 37°C was quickly mixed with 10 μl at 1 mM dye (DiO, DiI or DiD) solution or 30 μl at 5 mg/ml of TPCA-1 and the suspension was then incubated at 37°C for 30 min in a water bath. To remove free dye molecules, the suspension was centrifuged at 100, 000g twice and the pellet was suspended in HBSS. The suspension was extruded through a membrane with 0.2 μm pores using an extruder (Avanti polar lipids, Inc., Alabaster, AL) to make a uniform size of vesicles. Erythrocyte vesicles were similarly prepared.

2.4 Quantification of proteins and DNA

After cells were disrupted using nitrogen cavitation, the product after each centrifugation was quantitatively analyzed. The concentrations of proteins and double strain DNA were determined by BCA assay and QuantiFluor® dsDNA systems, respectively.

2.5 Efficiency of generating membrane-formed nanovesicles

We weighed the total cell lysate and the final vesicles after lyophilization, respectively. In particular, we had 66 mg of total cell lysate and 1 mg of cell membrane-formed vesicles after subtracting the buffer, so the vesicles had 1.5% of total cell mass. It was reported that the plasma membrane makes up of 2–3% of the total cell mass (20), thus 50–75% of cell membrane was successfully used to generate the vesicles using our nitrogen cavitation. After we quantified proteins of the vesicles using BCA assay, we obtained 0.5 mg of proteins in the vesicles. It means that 50% of proteins in vesicles, consistent with a plasma membrane composition (approximately 50% lipids and 50% proteins) reported in the literature (21).

2.6 Vesicle size and Zeta potential

The nanovesicles were characterized using dynamic light scattering (DLS) and TEM. The particle sizes and Zeta potentials were measured by Malvern Zetasizer Nano ZS90 (Westborough, MA). For Cryo-TEM, a drop of vesicle solution (1 mg/ml) was deposited on a carbon-coated grid discharged by PELCO easiGlow. After soaked by a piece of filter paper, the grid was quickly dropped in liquid nitrogen and stored overnight. The vesicles were imaged using TF20 TEM with a liquid nitrogen stage.

2.7 Endothelial cell culture and their activation after TNF- α challenge

Human umbilical vein endothelial cells (HUVECs) were cultured in the EBM medium supplemented with a kit including FBS, rhEGF, hydrocortisone, GA-100, bovine brain extract and ascorbic acid. To activate the expression of ICAM-1, 100 ng/ml TNF- α was added into the medium and at the defined time points the ICAM-1 expression was determined by Western blot.

2.8 Nanovesicle binding to HUVECs and their uptake

HUVECs at a concentration of 1.5×10^5 /well were seeded on a cover slip in a 12-well plate and after TNF- α treatment 60 μ l of vesicles at 2 mg/ml were added into each well and incubated for 35 min under continuous agitation to mimic as much as possible in vivo conditions. Cells were washed twice with PBS and fixed with 4% PFA for 10 min on ice. After washed twice with PBS, the cells were mounted on a slide with a mounting reagent containing DAPI (Life technologies, Grand Island, NY) and imaged using a confocal microscope (Olympus Fluorview FV1000). To quantitatively analyze the binding of nanovesicles to HUVECs and their uptake, at an equal fluorescence intensity HL60 nanovesicles or erythrocyte nanovesicles were added and incubated with HUVECs, 4 hr after treatment with 100 ng/ml of TNF- α . The cells were collected and analyzed by a flow cytometer (Accuri 6, BD, USA). The mean fluorescence intensity was measured to represent the uptake of nanovesicles in cells after subtracting the background. In binding experiments, we first incubated nanovesicles with TNF- α -treated HUVECs on ice for 15 min, and then washed cells with HBSS on ice. The temperature increased to 37 °C and the cells were incubated for 15 min for nanovesicle uptake. The cells were collected and analyzed by a flow cytometer. Similarly, the blocking experiment using ICAM-1 monoclonal antibody (10 μ g/ml) was performed.

2.9 Drug loading efficiency in nanovesicles

The TPCA-1-loaded vesicles were mixed with methanol to extract TPCA-1, and the concentration of TPCA-1 was measured using high-performance liquid chromatography system (Waters, Milford, MA). The drug was separated using a BEH C18 column (50 mm $\times$ 2.1 mm) and detected at a wavelength of 310 nm. The peak of TPCA-1 was confirmed by a G2-S Q mass spectrometer. The flow phase was prepared with 30% methanol in water and the flow speed was set at 0.5 ml/min.

2.10 Stability of nanovesicles as a drug carrier

The nanovesicle sizes were monitored over time using DLS. In the pharmaceutical application, we addressed whether lyophilization approach can maintain nanovesicle properties. In the study, we added 4% of sorbitol (commonly used in cryoprotectant additive) in nanovesicle suspension during the lyophilization. The drug loading stability in nanovesicles was evaluated using HPLC after a period of nanovesicle storage at 4°C. To determine the leakage of drug in nanovesicles, we centrifuged the suspension at 100,000g, collected the supernatant to quantify the drug content using HPLC.

2.11 ICAM-1 expression on HUVECs after treatment with TPCA-1 loaded nanovesicles

3h after treatment with TNF- α (100 ng/ml), HUVECs were incubated with 450 ng/ml of TPCA-1 loaded in HL 60 cell-or erythrocyte-membrane vesicles, in the presence of TNF- α (100 ng/ml). 4 h later the cells were harvested and lysated to analyze ICAM-1 expression using Western blot.

2.12 Mice Experiments

Adult CD1 mice (25–32 g) were purchased from Harlan Labs (Madison, WI). The mice were maintained in polyethylene cages with stainless steel lids at 20°C with a 12-h light/dark cycle and covered with a filter cap. Animals were fed with food and water *ad lib*. The Washington State University Institutional Animal Care and Use Committee approved all animal care and experimental protocols used in the studies. All experiments were made under anesthesia using intraperitoneal (i.p.) injection of a mixture of ketamine (120 mg/kg) and xylazine (6 mg/kg) in saline.

2.13 Intravital microscopy of live mouse cremaster venules

Using intravital microscopy, we real-time visualized how cell membrane-formed nanovesicles interacted with inflamed vasculature in a live mouse. TNF- α (500 ng in 250 μ l saline) was intra-scrotally injected into a mouse to cause vascular inflammation where endothelium highly expressed ICAM-1. 3 h after TNF- α injection, the mouse was anesthetized with i.p. injection of a mixture of ketamine (120 mg/kg) and xylazine (6 mg/kg), and maintained at 37°C on a thermo-controlled rodent blanket. A tracheal tube was inserted and a right jugular vein was cannulated for infusion of nanovesicles, or antibodies. After the scrotum was incised, the testicle and surrounding cremaster muscles were exteriorized onto an intravital microscopy tray. The cremaster preparation was superfused with thermo-controlled (37°C) and aerated (95% N₂, 5% CO₂) bicarbonate-buffered saline throughout the experiment. Images were recorded using a Nikon A1R⁺ laser scanning confocal microscope with a resonant scanner. To study the adherence of nanovesicles to an inflamed venule whose size was from 20–30 μ m, we simultaneously infused Alex Fluor-647 anti-mouse CD31 antibody (2.5 μ g per mouse) and HL 60 vesicles–labeled with DiO (0.1 mg/mouse) into the TNF- α -treated mouse, water immersion objective with NA=1.1 was used to image cremaster venules. Two lasers at 640nm and 488nm simultaneously excited cremaster tissues to image venules and vesicles at 10 frames/s for 512×512 pixels. Images were analyzed using Nikon software. To compare the adherence of HL 60 membrane vesicles to inflamed venules with erythrocyte nanovesicles, we simultaneously infused HL

60 vesicles labeled with DiL (560 nm) and erythrocyte vesicles labeled with DiO (488 nm) at the same concentration in a mouse. The intravital images were quantified using Nikon software (NIS Elements) to measure fluorescence intensity of vesicles. To address whether vesicles interact with resting endothelium, we injected HL 60 and erythrocyte nanovesicles via a tail vein to a mouse without TNF- α treatment. 1 h after injection, we exposed cremaster tissue under an intravital microscope to image these nanovesicles in cremaster venules. To examine whether neutrophils interact with nanovesicles, we labeled neutrophils using Alex-fluor-488-anti-Gr-1 antibody (1.5 μ g/mouse).

2.14 Bio-distribution of HL 60 cell membrane-formed nanovesicles

3 h after tracheal injection of LPS (10 mg/kg), mice were injected with HL 60 vesicles fluorescently-labeled with DiD (640nm/670nm) via a tail vein. 1 or 10 h later, we collected tissues including heart, spleen, lung, kidney and liver. To quantify the content of DiD, we homogenized the tissues and measure fluorescence intensity.

2.15 Mouse acute lung inflammation (ALI) model

CD1 male mice were used in this study. ALI was induced by tracheal spray of LPS (10 mg/kg) using a Model ICA-IC-M Micro Sprayer Aerosolizer (PenCentury Inc.). 3 h after LPS was challenged, mice were i.v. injected with HBSS, free drug (TPCA-1), TPCA-1 loaded erythrocyte vesicles and TPCA-1 loaded HL 60 vesicles at two doses of 0.33 and 1 mg/kg, respectively.

2.16 Bronchoalveolar lavage fluid collection and cell counts

At 13 h post-LPS administration, mice were anesthetized with i.p. injection of ketamine and xylazine mixture. The trachea was cannulated, and 1 ml HBSS was intratracheally infused and withdrawn to obtain lavage fluid. This procedure was repeated twice. The bronchoalveolar lavage (BAL) fluid was centrifuged at 420 g for 4 min, and cell pellets were suspended in 0.7 ml red blood cell lysis buffer (Qiagen, Valencia, CA). 30 min later, the cells were pelleted by centrifugation at 420 g for 4 min, and suspended in 0.5 ml HBSS. The total cell number was determined with a hemocytometer. Cell suspensions were diluted to a final concentration at 1×10^5 cells/ml and a 200- μ l of the suspension was spun onto a slide at 700 rpm for 5 min using a cytocentrifuge (Shandon, Southern Sewickley, PA). The slides were stained with Diff-Quick dye, and examined at a magnification of 400 by light microscope. The percentage of neutrophils were determined after counting 200 cells in randomly selected fields.

2.17 Cytokines and lung vascular permeability

Cytokine levels in BALF were determined using commercial ELISA kits for TNF- α and IL-6 (Biolegend, San Diego, CA) according to the manufacturer's instructions. The triplicate experiment was conducted. The permeability was evaluated via the lavage protein content change. The total protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL).

2.18 Statistical Analysis

Data are expressed as mean $\pm$ SD. Statistical analysis was conducted using student T-test in Origin 8.5. p values < 0.05 are considered significant. p values < 0.01 are considered extremely significant.

3. Results

Here, we developed a general method to create cell membrane-formed vesicles using nitrogen cavitation (22) which instantly disrupts cell membrane and maintains intact biological functions of membrane antigens (Fig. 1A). In this study, we used HL 60 cells as a model because their functions are similar to neutrophils *in vivo* (23), and chose erythrocytes (red blood cells) as negative control. After HL 60 cells were disrupted by nitrogen cavitation under a high pressure of 350 psi, the resulting solution was composed of membrane-formed vesicles, intracellular molecules and nucleus. Using a differential centrifugation approach we purified the needed vesicles. A composition of the suspension after each centrifugation was determined using protein and DNA assays (Fig. 1B). A pellet after 2,000 g showed that the majority was DNA with a 70 % of nuclear molecules, but only 10% of proteins. The supernatant was further centrifuged at 100,000 g. The resulting supernatant showed nearly 88 % of proteins and 30% of DNA of a whole cell lysate. In contrast, the pellet after 100,000 g centrifugation is unlikely to contain DNA molecules, but 1.3% of whole cell proteins existed in the pellet. The pellet was weighed after lyophilization and the proteins were quantified using BCA assay. It was found that 50% of the pellet mass was proteins (see Methods), consistent with the studies in which the cell plasma membrane is comprised of approximately 50% lipid and 50% protein by weight (21). The result suggests that the pellet is mainly comprised of cell plasma membrane. We also found that 50–75% of HL60 cell plasma membrane was successfully exploited to form vesicles (Methods), so our approach is highly efficient to produce cell membrane-formed vesicles. Using dynamic light scattering we measured the sizes of the products after each centrifugation (Fig. S1), showing a broad range of particle sizes. After extruded through a membrane with a pore size of 200 nm, the vesicles gave rise to the uniform size (Fig. S1). Because the nanovesicle size is dependent on the membrane pore size, it is possible to make different size of nanovesicles using the different pore size of membrane.

Using cryo-TEM and dynamic light scattering (Fig. 1C, D and Fig. S2) we characterized nanovesicles. Cryo-TEM clearly showed a shell structure with size of 200 nm in diameter, and the thickness of vesicles is 3–4 nm, which equals to that of cell membrane (24) (Fig. 1C). The result is consistent with the composition of nanovesicles as discussed above. The polydispersity of nanovesicles was studied using dynamic light scattering (DLS) (Fig. S3). The result showed polydispersity index (PDI) is 0.25, indicating that nanoparticles appeared moderate uniform, consistent with Cryo-TEM images. The zeta potentials of HL60 cell membrane-formed nanovesicles (HV) (–16 mV) was close to their parent cells (–14 mV) (Fig. 1E). Similarly, it was observed for erythrocyte membrane-formed vesicles (EV) (Fig. 1D and E). Thus, the nanovesicles made using nitrogen cavitation originated from their source cell membranes.

We also investigated the size change over time using DLS (Fig. S4), and we observed that the HV nanovesicles increased their size by 10% 6 days after nanovesicles were stored at 4 °C. For the pharmaceutical application, we also addressed whether the lyophilization approach can maintain the nanovesicle size. When we lyophilized nanovesicle suspension, we added 4% of sorbitol (commonly used additive). When we reconstructed the lyophilized nanovesicles, the size of nanovesicles dramatically increased compared with their original size. However, after sonicated, the nanovesicles became 200 nm which is same to that prior to lyophilization (Fig. S5). The result indicates that our nanovesicles are similar to liposomes which can be stored as lyophilized formulation. Furthermore, we addressed the drug retention in nanovesicles using HPLC. After TPCA-1-loaded nanovesicle suspension was stored at 4°C for a period of time, the nanovesicle suspension was centrifuged at 100,000 g to separate released drug from nanovesicles, and quantified the released drug using HPLC (Fig. S6). While in initial 2 days 30% of drug was released from nanovesicles, no drug was further released in a week of storage at 4 °C. The studies show that nanovesicles contained the drug for a long period time. Our nanovesicles can be stored after lyophilization, therefore the drug release in nanovesicle suspension could be prevented.

DMSO (dimethyl sulfoxide) treatment can promote HL 60 cells to differentiate and highly express integrin β_2 (Supplementary Fig. S7) which plays a central role in neutrophil functions (23), such as adherence to endothelium. We also studied a profile of proteins in the products after each centrifugation using SDS-PAGE and Western blots (Fig. S8 and S9). The HV nanovesicles showed a similar protein pattern in SDS-PAGE to HL 60 cell lysate and the supernatant after centrifugation at 100,000 g. In Western blot, integrin β_2 on HV nanovesicles was enriched compared to the whole cell lysate, but the intracellular protein (actin) was not detected in HV nanovesicles at the same loading amount of samples (Fig. S9). This is correlated to the cryo-TEM image (Fig. 1C) and analysis of HV nanovesicle compositions (Fig. 1B). We further quantitatively analyzed integrin β_2 on HV nanovesicles. We found that HV nanovesicles highly expressed integrin β_2 and showed a large ratio of integrin β_2 to actin (intracellular protein) compared to their source cells (Fig. 2A and Fig. 2B). This enrichment might be due to the nanovesicle formation leading to an increase of integrin β_2 density. In contrast, erythrocytes and their vesicles did not express integrin β_2 .

To study whether HV nanovesicles can bind to activated endothelium, we fluorescently labeled vesicles with lipid dyes. HUEVCs (human umbilical vein cells) dramatically expressed ICAM-1 after TNF- α treatment (100 ng/ml) (Supplementary Fig. S10), and then were incubated with HV or EV nanovesicles. The confocal images (Fig. 2C) of inside cells showed that HV nanovesicles were more efficiently internalized by HUVECs compared with EV nanovesicles. To quantitatively analyze the nanovesicle binding to HUVECs and their uptake, flow cytometry was used to measure the mean fluorescence intensity of vesicles per cell (Fig. 2D). After nanovesicles were incubated with HUVECs at 37 °C the mean fluorescence intensity of vesicles was measured using flow cytometry. The result showed that the uptake of HV nanovesicles by HUVECs increased by 3-fold compared with that of EV nanovesicles. However, this study cannot exclude nonspecific binding between ICAM-1 and integrin β_2 to contribute the nanovesicle uptake because we still observed the uptake of EV nanovesicle by HUVECs. To address this question, we performed the binding experiment. We incubated nanovesicles with TNF- α -treated HUVECs on ice for 15 min.

After washing we increased the temperature to 37 °C to initiate bound nanovesicles to be internalized (Fig. 2D). It is noted that EV nanovesicle uptake was dramatically inhibited, but HV nanovesicles were not. When we blocked ICAM-1 binding to integrin β_2 using anti-ICAM-1 antibody, the HV nanovesicles significantly decreased their uptake by HUVECs, clearly indicating that the binding of integrin β_2 to ICAM-1 plays a central role in mediating the uptake of HV nanovesicles in HUVECs.

To examine whether HV nanovesicles interacted with inflamed vessels *in vivo* we performed intravital fluorescence confocal microscopy of live mouse cremaster venules (25). 3 h after intrascrotal (i.s.) injection of TNF- α , which causes the marked expression of ICAM-1 on endothelium vessels (25), we observed that intravenously (i.v.) infused HV nanovesicles were adherent to cremaster venules fluorescently labeled by anti-CD31 (PECAM-1), a marker of endothelium (26) (Fig. 3A and Movie 1 showing a 3D image). To define whether integrin β_2 is required for the adherence of HV nanovesicles to activated endothelium, we simultaneously infused HV and EV nanovesicles into the TNF- α -challenged mouse. We observed many puncta of HV nanovesicles adherent to venules compared to EV nanovesicles (Fig. 3B and Movie 2). However, in a mouse without TNF- α treatment (no inflammation), we observed neither of HV nor EV nanovesicles were adherent to the vessel wall (Fig. 3C and Movie 3). After analyzing fluorescent intensity of HV and EV nanovesicles, we quantified adherent nanovesicles per field of intravital images (Fig. 3D). We found that integrin β_2 is a determinant for binding of HV nanovesicles to activated endothelium. The endothelial vessel is so thin that it is difficult to determine whether nanovesicles were internalized in the endothelium under an intravital microscope. However, in a 2–3 h period of observation using intravital microscopy the adherent nanovesicles rarely detached from vessel walls, implying that the adherent nanovesicles were more likely to be internalized by endothelium. We also observed some puncta that were larger than the physical size of nanovesicles (Fig. 3A and Fig. 3B). This might be related to the infusion of HV nanovesicles with large organelles in the endothelium after nanovesicle internalization. Another possibility may be relevant to multiple nanovesicles closely adherent to endothelium because of a heterogeneous distribution of adherent molecules *in vivo*.

At inflammation sites, neutrophils are adherent to vessel walls, so it is unknown whether HV nanovesicles interact with neutrophils. We simultaneously infused Alex-fluor-488-labeled anti-Gr-1 antibody to mark neutrophils (25), and fluorescently-labeled HV nanovesicles in a mouse. The HV nanovesicles did not interact with neutrophils, but accumulated in inflamed vessels close to adherent neutrophils (Fig. 3E and Movie 4).

Next, we studied a bio-distribution of HV nanovesicles in the mice with or without lung LPS instillation at 1 and 10 h after i.v. injection of the nanovesicles (Fig. S11A and S11B). The tissue deposition of HV nanovesicles dramatically increased in inflammatory lungs compared to healthy ones. We calculated the ratios of HV nanovesicles accumulated in lung over in liver, finding that the vesicle accumulation in inflammatory lungs increased by 5–10 folds compared to health ones (Fig. 3F). The nanovesicle tissue deposition in LPS-challenged mice did not change with time (Fig. 3F), suggesting that the vesicles were internalized by endothelial vessels, consistent with the results of intravital microscopy (Fig. 3A and 3B).

To examine whether HV nanovesicles as a carrier were able to deliver therapeutics to inflamed vasculature impairing vascular inflammation, the vesicles were loaded with TPCA-1 (2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide), which is a NF- κ B inhibitor (27) and its loading efficiency was quantified using high-performance liquid chromatography (Fig. S12). The NF- κ B pathway is a central regulator to control inflammation responses when infection or tissue damage occurs. After HV or EV nanovesicles loaded with TPCA-1 were incubated with TNF- α -activated HUVECs, HV nanovesicles dramatically reduced ICAM-1 expression on endothelial cells (Fig. 4A).

Acute lung inflammation and injury by infection or tissue damage quickly morph into its most severe form, acute respiratory distress syndrome (ARDS) which causes 40% mortality in annual 200,000 incidences in the United States (11). The pathological underlying acute lung inflammation is primarily linked to cytokine storms produced by resident lung macrophages leading to the activation of endothelium that recruits neutrophils into the lung (28). The NF- κ B pathway is a central regulator to activate endothelium to express ICAM-1 for neutrophil recruitment and cytokine storms, so we examined whether HV nanovesicles loaded with TPCA-1 could alleviate lung vascular inflammation. 3 h after instillation of LPS into a mouse lung, we i.v. administered HV, EV nanovesicles or free drug, respectively. The therapeutic effects at 13 h after LPS challenge were investigated. Infiltration of neutrophils in the lung dramatically reduced after the administration of HV nanovesicles compared with EV nanovesicles or free drug and it was dose-dependent (Fig. 4B). Similarly, the lung permeability decreased (Fig. 4C), representing that HV nanovesicles can improve the lung integrity, thus preventing lung injury from edema (29). Interestingly, even at a low dose of 0.33 mg/kg, HV nanovesicles showed a marked reduction of infiltrated neutrophils and lung permeability, but EV nanovesicles and free drug rarely showed the bio-action. This result strongly indicates that endothelial targeting of HV nanovesicles enhanced the therapeutic efficacy. The inflammatory factors, such as TNF- α and IL-6 decreased after the treatment with HV nanovesicles at 1 mg/kg of TPCA-1 compared with EV nanovesicles or free drug (Fig. 4D and 4E) showing that the lung inflammation attenuated. But at a dose of 0.33 mg/kg, the effect of HV nanovesicles on TNF- α and IL-6 did not show a greater benefit than EV nanovesicles, which might be associated with the limitation of drug dose required for TPCA-1 to inhibit the NF- κ B pathway in endothelium to affect inflammatory factor levels in the lung.

4. Discussion

Intercellular communication is a hallmark of multicellular organisms and can be mediated through direct cell-cell contact or transfer of secreted molecules. Recently, extracellular vesicles were discovered as the third mechanism for intercellular communication (30). Extracellular vesicles are cell-derived membrane vesicles secreted by many cell types *in vitro*, but also present in body fluids, secreted by cells *in vivo* (30). There are two types of membrane vesicles of endosomal and plasma membrane origin called exosomes and microvesicles, respectively. Proteomic and transcriptomic profiling of extracellular vesicles show that they are natural vehicles of protein, mRNA and miRNA transport between cells (31, 32). These findings gave rise to a possibility that the vesicles could be explored for delivery of exogenous therapeutic cargos *in vivo* (33). Advantages of extracellular vesicle-

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based drug delivery systems over synthetic nanoparticle delivery systems are their natural targeting properties (33), their natural stability in blood due to evasion of complement and co-agulation factors and their immune-tolerance in the case of patient-derived extracellular vesicles. Despite the exciting progress with the discovery of the drug delivery potential of extracellular vesicles, there exist several challenges in the clinical translation of the research findings (31). Given studies have shown that extracellular vesicles mediate the spread of pathology. Endogenous cargos in extracellular vesicles contain signaling proteins and genetic molecules which promote the unwanted side effects. Extracellular vesicles are complex and heterogeneous so it is difficult to predict their biological functions as drug carriers. In addition, clinical translation is hindered by the lack of suitable and scalable technologies for the generation and purification of extracellular vesicles. Thus, novel methods are needed to make pharmaceutically controllable and homogeneous membrane vesicles for targeted drug delivery.

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Here we report on the generation of neutrophil cell-membrane-formed nanovesicles using nitrogen cavitation. The studies (Figure 1) show that these nanovesicles possess the source cell membrane features, such as cell membrane antigens. In addition, we have shown that these vesicles are capable of targeting activated endothelial cells *in vitro* and *in vivo* (Figure 2 and Figure 3). Thus, our nanovesicles would appear the similar functions to extracellular vesicles secreted by cells, particularly for microvesicles, but our nanovesicles show the novel properties for drug delivery.

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Nitrogen cavitation is a novel approach to rapidly break cells and maintain biological functions of membrane antigens. In a vessel container, nitrogen disperses in a cell under a high pressure. When the gas pressure is rapidly released, the nitrogen comes out the solution to form bubbles within the cell and the bubbles expand, breaking the cell wall and quickly releasing the cell contents. Simultaneously the cell plasma membrane spontaneously forms many vesicles. Using the nitrogen cavitation approach, we have demonstrated that we can make controllable and producible membrane nanovesicles without genetic materials which are the safety concerns for drug delivery. Importantly, this approach is easy to scale up to make large quantities of nanovesicles for clinical applications. Chemical agents (cell lysis) or mechanical forces (a homogenizer) were used to make red blood cell membrane-coated nanoparticles (34) and stem cell-derived “nanoghosts” (35). Compared with those approaches, our nitrogen cavitation approach is not subjected to chemicals or long-term physical stress which could change biological functions of membrane antigens of interest.

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Exploiting of cell membrane materials for drug delivery represents a unique top-down approach that offers the advantage of being able to completely replicate the surface antigenic diversity of parent cells. Here we have successfully created neutrophil-membrane-formed nanovesicles possessing integrin β_2 which binds to ICAM-1 highly expressed on endothelial vessels during inflammation. Using intravital fluorescence microscopy of cremaster venules, we have demonstrated that neutrophil-derived nanovesicles selectively and specifically bind to activated endothelial vessels and subsequently are internalized (Figure 3). In contrast, normal endothelial vessels interact with neither neutrophil-nor red blood cell-derived nanovesicles. In the acute lung inflammation model, neutrophil nanovesicles dramatically accumulate in the inflamed lungs compared with the healthy lungs. When administration of

TPCA-1-loaded neutrophil-derived nanovesicles, they impaired the lung inflammation and prevented lung edema. Compared with the control of EV nanovesicles, the result clearly showed that neutrophil membrane-derived nanovesicles are a novel drug delivery platform for targeting and treating vascular inflammation disorders. Recently it was reported that red blood cell (RBC) membrane was coated to PLGA(poly(lactic-co-glycolic acid) nanoparticles and the core-shell nanoparticles can increase the blood circulation time and were applied for detoxication (34). This concept of core-shell platforms would allow us to load a wide array of therapeutic cargos in neutrophil-membrane nanovesicles. Because of the antigen diversity on neutrophil membrane, the core-shell structured neutrophil nanovesicles would have advantages over anti-ICAM-1-conjugated nanoparticle delivery systems for vascular targeting (16).

Neutrophil-membrane-formed nanovesicles can be applied to developing personalized nanomedicines. While we used HL 60 cells, as model cells to make nanovesicles, these neutrophil-like cells possess human neutrophil functions *in vivo*, therefore we expect that we would translate the nitrogen cavitation approach to make similar nanovesicles using human neutrophils. The nitrogen cavitation approach was applied to human neutrophils for the isolation of membrane proteins (22), and the studies are consistent with our report here. Because antigens expressed on membrane are key components for targeted drug delivery, we believe that when we translate our technology to human neutrophils, nanovesicles would retain the membrane properties of parent cells. Human neutrophils account 50–70% of all circulating leukocytes (36), thus using the highly efficient nitrogen cavitation approach developed here (50–75% of cell membranes used to form nanovesicles), it would be easily to generate large quantities of nanovesicles from individual blood samples. After loading of therapeutic cargos into nanovesicles, they are given back to the original donors. This strategy is attractive because of no concerns of immunotoxicity in patient-derived nanovesicles.

Here we describe a general method to generate cell-membrane-formed nanovesicles using nitrogen cavitation. It is applicable to all types of cells including cancer cells, platelets, endothelial and epithelial cells. Intercellular interactions are underlying components of pathogenesis of most diseases. After identifying key cell types in disease tissues, they could be utilized as a building block to generate cell membrane-formed nanovesicles which could selectively target disease sites. Our studies presented here have revealed a proof-of-concept of developing drug delivery platforms driven by a given disease. The disease-driven strategy presents a new and novel top-down approach for designing drug delivery systems potentially used to treat a wide range of diseases, such as inflammation disorders (5, 7) and cancer (8).

5. Conclusions

In summary, we have presented a novel approach to create a nanovesicle-based drug delivery platform by employing activated neutrophils as a building block to generate cell membrane-formed nanovesicles. These nanovesicles replicate the membrane features of the source cells, are able to target inflamed vasculature, and dramatically attenuate acute lung inflammation and injury. The nitrogen cavitation approach is a powerful tool to generate nanovesicles retaining intact targeting molecules and easy to scale up for clinical

applications. Human neutrophils are the most rich of leukocyte in blood, thus we could develop personalized nanomedicines using human neutrophils. In general, our studies have demonstrated a new top-down strategy for designing nanomedicine driven by a given disease and employing diseased tissues to produce disease-targeted nanovesicle-based therapeutics. This concept could be applied in a wide range of disease models

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported by NIH grant K25HL111157 and 1R01GM116823, and in part by the Health Sciences and Services Authority of Spokane (HSSAS) to Z. W.. Thanks Drs. L. Wang and M. Li from the Department of Biological Structure in the University of Washington for assisting in cryo-TEM.

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Figure 1. Generation of cell-membrane-formed nanovesicles and their characteristics

(a) Schematic shows a process to generate a uniform size of nanovesicles including cell disruption, differential centrifugation and extrusion. (b) Quantification of DNA and proteins contained in each step of centrifugation as described in (a). (c) Cryo-TEM of cell membrane-formed vesicles of HL 60. (d) Average sizes of HL 60 cell membrane-formed vesicles (HV) and erythrocyte vesicles (EV) using dynamic light scattering. (e) The zeta potentials of HL 60 and erythrocyte cells and their nanovesicles. All data expressed as Mean $\pm$ SD, n=3–6.

Figure 2. Integrin β_2 on HV nanovesicles increases their binding to HUVECs and their internalization

(a) Western blot of HL 60 and erythrocyte cells and their nanovesicles. (b) The integrin β_2 expression against actin in HL 60 cells and in their nanovesicles quantified from Western blot. (c) Fluorescence confocal images of inside HUVECs after incubated with Dil-fluorescently-labeled nanovesicles (HV or EV). HUVECs were treated with 100 ng/ml of TNF- α . (d) Uptake of HV or EV nanovesicles by HUVECs obtained from flow cytometry. 3 h after HUVECs were treated with TNF- α (100 ng/ml), they were incubated with Dil-fluorescently-labeled vesicles, and then the flow cytometry was used to measure the mean

fluorescence intensity per cell. The binding of nanovesicles to HUVECs was performed at 0 °C and followed with washing and the temperature increase to 37 °C. To inhibit the binding of integrin β_2 to ICAM-1, the HUVECs were pre-treated with anti-ICAM-1 antibody (10 μ g/ml). ** represents p value < 0.01. All data expressed as Mean $\pm$ SD.

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Figure 3. Activation of endothelium vessels is required for HV nanovesicles adherent to the vessels, resulting in enhanced vesicle deposition in inflammatory lungs

(a) Intravital images of a cremaster venule of a live mouse intrascrotally treated with TNF- α (0.5 μ g) after i.v. infusion of DiO-fluorescently-labeled HV vesicles (green) and Alex-Fluor-647-labeled anti-CD31 (pink). The image was taken by lasers at 488 nm and 640 nm using A1R⁺ resonant-scanning confocal microscope. Intravital images of cremaster venules activated with (b) or without TNF- α (c) after i.v. infusion of DiL-fluorescently-labeled HV and DiO-fluorescently-labeled EV nanovesicles at 0.1 mg/mouse, respectively. In (c) 1h after tail vein injection of the vesicles, intravital microscopy was performed. (d) Quantification of the nanovesicles adherent to cremaster venules based on intravital images using the Nikon software (NIS Elements). The vessel size was from 20–30 μ m in diameter. (3 mice per group). $p < 0.003$. (e) Intravital image of HV nanovesicles accumulated in an inflamed area where neutrophils existed. Alex-fluor-488-anti-Gr antibody (green) and Dil-fluorescently-labeled HV nanovesicles (red) were simultaneously i.v. injected to a live mouse intrascrotally treated with TNF- α . The white lines show vessel walls. (f) Ratios of HV nanovesicle deposition in lung over in liver of mice with or without (w/o) lung LPS

instillation. 1 and 10 h after i.v. injection of nanovesicles, the tissues were collected to analyze DiD fluorescently labeled to HV nanovesicles. *, and ** represent p value <0.05 and 0.01. (3–4 mice per group). All data expressed as Mean $\pm$ SD.

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Figure 4. TPCA-1-loaded HV nanovesicles attenuate vascular inflammation *in vitro* and *in vivo* (a) ICAM-1 expression of TNF- α -treated HUVECs after incubated with TPCA-1-loaded HV nanovesicles (HV-TPCA-1) and TPCA-1-loaded EV nanovesicles (EV-TPCA-1) respectively. Western blot shown in the inset. The diagram shows the experimental protocol for *in vivo* studies. Numbers of neutrophils (b), and the concentrations of proteins (c), TNF- α (d) and IL-6 (e) in BALF 10 h after i.v. injection of HBSS, TPCA-1 solution, EV-TPCA-1 and HV-TPCA-1 vesicles in mice 3 h after LPS challenge (10mg/kg). The dose of TPCA-1 is 0.33 mg/kg and 1 mg/kg respectively. All data expressed as mean $\pm$ SD (3–4 mice per group). *, **, and *** represent p value <0.05, 0.01 and 0.001 in two-way t-Test.