Video Article

Generation of Lymphocytic Microparticles and Detection of their Proapoptotic Effect on Airway Epithelial Cells

Chun Yang^{*1}, Wei Xiong^{*2}, Qian Qiu², Houda Tahiri¹, Carmen Gagnon¹, Guoxiang Liu², Pierre Hardy¹

¹Departments of Pediatrics and Pharmacology, University of Montréal

²Department of Pulmonology, Chongqing Southwest Hospital, Third Military Medical University

*These authors contributed equally

Correspondence to: Pierre Hardy at pierre.hardy@recherche-ste-justine.qc.ca

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Abstract

Interest in the biological roles of cell membrane–derived vesicles in cell–cell communication has increased in recent years. Microparticles (MPs) are one such type of vesicles, ranging in diameter from 0.1 μ m to 1 μ m, and typically shed from the plasma membrane of eukaryotic cells undergoing activation or apoptosis. Here we describe the generation of T lymphocyte–derived microparticles (LMPs) from apoptotic CEM T cells stimulated with actinomycin D. LMPs are isolated through a multistep differential centrifugation process and characterized using flow cytometry. This protocol also presents an *in situ* cell death detection method for demonstrating the proapoptotic effect of LMPs on bronchial epithelial cells derived from mouse primary respiratory bronchial tissue explants. Methods described herein provide a reproducible procedure for isolating abundant quantities of LMPs from apoptotic lymphocytes *in vitro*. LMPs derived in this manner can be used to evaluate the characteristics of various disease models, and for pharmacology and toxicology testing. Given that the airway epithelium offers a protective physical and functional barrier between the external environment and underlying tissue, use of bronchial tissue explants rather than immortalized epithelial cell lines provides an effective model for investigations requiring airway tract tissue.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52651/>

Introduction

Microparticles (MPs) are biologically active submicron membrane vesicles released following cell activation or apoptosis. MPs are derived from both healthy and damaged cells and are implicated in many physiological and pathological processes.¹ MPs have been detected not only in human plasma, but also in inflammatory and apoptotic tissue. The biological utility of cell membrane–derived MPs has been demonstrated in various settings, including cell signalling models and as pharmacological tools.^{2,3} We previously demonstrated that LMPs derived from T lymphocytes following actinomycin D stimulation (to induce apoptosis) suppress angiogenesis and inhibit endothelial cell survival and proliferation.^{4,5} The antiangiogenic effects of LMPs may vary significantly depending on the stimuli used to activate T lymphocytes *in vitro*.⁶

The airway epithelium functions as a protective physical and functional barrier. Increased numbers of T lymphocytes in the airway can contribute to cell damage and airway inflammation.⁷ We have shown that LMPs induce apoptosis of human bronchial epithelial cells,⁸ which indicated LMPs may change barrier function of bronchial epithelium *in vivo*. Apoptotic cells can be identified using the TUNEL method, which detects *in situ* DNA fragmentation.

The overall goal of this protocol is to illustrate the *in vitro* production of LMPs from a T lymphocyte cell line, and to demonstrate their proapoptotic effect on airway epithelial cells. *In situ* cell death detection demonstrated that LMPs strongly induce airway bronchial epithelial cell death, suggesting that LMPs-mediated injury to the airway epithelium may impact barrier function of the damaged epithelium.

Protocol

NOTE: Male C57BL/6 mice (5–7 weeks old) are from Charles River Laboratories International, Inc. (St-Constant, Quebec, Canada.) and manipulated according to protocols approved by the CHU Sainte-Justine Animal Care Committee. Mouse bronchial tissue explants provide a good source of primary bronchial epithelial cells for investigating the proapoptotic effects of LMPs on epithelial cells. This protocol describes the *in vitro* generation of LMPs, as well as a method for detecting apoptotic epithelial cells on LMPs-treated bronchial tissue explants. This protocol consists of 3 sections.

1. LMPs Production and Characterization

NOTE: To prevent contamination, ensure that all materials used in this experiment are sterile or autoclaved. Perform all steps at RT in a biological safety cabinet under sterile condition, unless otherwise indicated.

1.1) Stimulation and Collection of MPs⁹

1. Thaw an aliquot of 10 million CEM T cells in a 37 °C water bath. Dilute in 10 ml pre-warmed serum-free hematopoietic medium such as X-VIVO, in a 15 ml sterile tube and centrifuge at 200 x g x 5 min. Aspirate the supernatant and resuspend cells in 5 ml pre-warmed medium.
2. Transfer cells into a T75 tissue culture flask (for suspension cells) with 15 ml pre-warmed hematopoietic medium such as X-VIVO and incubate for 4 days in a humidified incubator at 37 °C with 5% CO₂.
3. After 4 days, transfer all the culture medium and cells into a T175 tissue culture flask containing 100 ml fresh medium. Continue incubating the cells for about 72 hr under the same conditions until they have grown to a density of 2 million cells/ml.
4. Evenly split cells between four T175 flasks each containing 150 ml fresh medium and continue cell culture until cells have grown (approximately 48 hr incubation) to a density of 2 million/ml.
5. Collect cells from each flask by centrifugation at 200 x g for 5 min and resuspend 300 x 10⁶ cells into a new T175 flask containing 150 ml fresh medium, to maintain the 2 million/ml cell density.
6. Add actinomycin D (dissolved in DMSO at 2 mg/ml) to the medium at a final concentration of 0.5 µg/ml and incubate for 24 hr.
7. Transfer all the culture medium into 50 ml conical tubes and spin down the cells at 750 x g for 5 min. Transfer the supernatant into 50 ml conical tubes and centrifuge at 1,500 x g for 15 min to remove large cell fragments.
8. Transfer the supernatant into a 250 ml bottle and ultracentrifuge at 12,000 x g for 50 min. Discard the supernatant and collect pellets.
9. Wash LMPs-enriched pellets with 40 ml sterile PBS in a 50 ml tube by centrifugation at 12,000 x g for 50 min. Repeat this step twice.
10. Collect the last wash supernatant; it will be used as vehicle control. Suspend the LMPs pellets in 1 ml of PBS and transfer into a 1.5 ml sterile microtube. Aliquot and store isolated LMPs at -80 °C (to avoid multiple free-thaw cycles).

1.2) Characterization of MPs via FACS Analysis⁴

1. Prepare 2 samples of annexin buffer, 1 with and another without CaCl₂: Hepes 10 mM, NaCl 140 mM, plus or minus 5 mM CaCl₂.
2. Filter annexin buffer and FACS flow sheath fluid using a 0.22 µm filter to remove particles.
3. Dilute 1 µl of LMPs in 44 µl of annexin buffer with 5 mM CaCl₂ into a FACS tube. Prepare another tube with 1 µl of LMPs in 44 µl of annexin buffer without CaCl₂ (negative control).
4. Add 5 µl of annexinV-Cy5 in each tube and mix well. Incubate for 15 min at RT in the dark. Stop the reaction by diluting the mix with 400 µl of FACS flow sheath fluid in each tube.
5. Add 10 µl (200,000 beads) of 7 µm counting beads suspension as an internal standard in each tube to obtain an absolute count.
6. Establish gates of relative size (FSC-H, PMT E00, log scale) and relative granularity (SSC-H, PMT 325, log scale) dot plot on the flow cytometer using size-calibrated fluorescent beads of 1 µm (gate 1) and counting beads gate at 7 µm (gate 2).
7. Analyze the LMPs sample on FSC-H/SSC-H plot using the established gates and FL-4 channel for annexin (PMT 765, log scale) dot plot, by acquiring a signal until 20,000 counting beads are reached in gate 2.
8. Determine the positive annexinV events of LMPs in annexin buffer containing CaCl₂, and then subtract the events of LMPs in annexin buffer without CaCl₂ (negative control).
9. Calculate the absolute number of MPs based on the following equation:

$$\text{MPs count} = \frac{\text{No. of events in region containing MPs}}{\text{No. of events in absolute count bead region}} \times \frac{\text{No. of beads per test}}{\text{Test volume (}\mu\text{l)}}$$

1.3) Determination of MP Protein Concentration (Bradford Assay)

1. Prepare 5 serial dilutions of a protein standard from 1.25 to 20 µg/ml. Pipette 800 µl of each standard and sample solution into a clean test tube in duplicate. Add 200 µl of Bradford dye reagent to each tube. Mix well, then incubate at RT for 5 min.
2. Measure absorbance at 595 nm. Determine the protein concentration of LMPs using the linear regression of standard curve.

2. Bronchial Tissue Explants and LMPs Treatment

NOTE: Pay special attention to the sterile working environment, and aseptically prepare the solutions and medium used in following experiments. To prepare the Complete Healing Medium, add 1 ml of Tissue Healing Medium Supplements with Serum (thawed on ice) to 100 ml Tissue Healing Medium and mix well.

2.1) Preparation of Bronchial Tissue Explants

1. Before culturing, scratch 6 areas of 1 cm² each at the edge of the surface of each 100 mm tissue culture dish with a scalpel blade. Coat each scratched 100 mm tissue culture dish with 2 ml of the culture dish coating solution, and incubate the dish in a humidified CO₂ incubator O/N at 37 °C. Vacuum aspirate the surplus solution and fill the dish with 15 ml of Tissue Washing Medium.
2. Euthanize C57BL/6 mice (5 to 7 weeks old) by CO₂ inhalation according to protocols approved by the animal care ethics committee.
3. Aseptically dissect lung tissue with scalpel, Dumont super fine tweezer, and surgical scissors. Carefully remove parenchyma and blood vessels. Place lung tissue into ice-cold Tissue Washing Medium for transport to the laboratory, if applicable.
4. Further dissect bronchus submerged in the Tissue Washing Medium and separate the bronchus with a diameter of 1 to 2.5 mm from peripheral lung tissues. Slice bronchial tissues into ~5 mm thick bronchial rings with a scalpel.
5. Use a scooping motion with the sterile curved microdissecting forceps to pick up the bronchial fragments and place them onto the scratched areas of the dishes.
6. Remove the Tissue Washing Medium, and incubate the fragments at RT for ~5 min to allow them to adhere to the dishes.

7. Add 10 ml of Complete Healing Medium to each dish and place them in a controlled atmosphere modular incubator chamber. Flush the chamber with high O₂ gas mixture (70% O₂, 25% N₂ and 5% CO₂). Place the chamber in a benchtop orbital incubator and shake it at 37 °C. Shake the chamber for 24 hr at 10 cycles per minute to allow the medium to flow intermittently over the fragments.
8. After 24 hr incubation, observe the tissue explants under a phase-contrast inverted light microscope. Select bronchial explants with complete, fine hair movement and lively bronchial epithelium for subsequent LMPs treatment.

2.2) LMPs Treatment

1. Prepare complete growth medium as follows: thaw growth medium supplements with serum and fibroblast inhibitor on ice. Add 1 ml of the growth medium supplements with serum and 200 µl fibroblast inhibitor to 100 ml of growth medium; mix thoroughly. Warm the complete growth medium at 37 °C for 10 min prior to use.
2. Dilute isolated LMPs in a new sterile eppendorf tube with PBS to prepare a LMPs stock at a concentration of 800 µg/ml.
3. Add 0.5 ml of Complete Growth Medium to each well of a 12-well tissue culture plate.
4. Transfer the selected bronchial explants with the curved microdissecting forceps from the previous protocol (section 2.1) to each well of the tissue culture plate.
5. Label the culture plate appropriately to identify LMPs treatment wells and control wells. Add 25 µl LMPs stock into each LMPs treatment well (for a final concentration of 40 µg/ml) and 25 µl control vehicle (see LMPs production) to the control wells.
6. Continue the incubation in a controlled atmosphere modular incubator chamber at 37 °C with gentle shaking.
7. After 24 hr, wash explants 3 times with PBS and proceed to the next step (4% paraformaldehyde [PFA] fixation).

3. Histopathological Examination

3.1) Prepare the Following Solutions Before Proceeding to the Next Steps

1. Prepare 1x PBS buffer by mixing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4.
2. To prepare 4% PFA, dissolve 20 g of PFA in 400 ml of water, heated at 60 °C with stirring; add a few drops of 10 M NaOH to clear the solution. Next add 1x PBS buffer and adjust the volume to 500 ml and pH to 7.4. Filter and aliquot; store at -20 °C.
3. Prepare the following dehydration or rehydration reagents; 100%, 90%, 70%, 50% ethanol and xylene.

3.2) Explant Fixation and Tissue Section Deparaffinization

1. Place each explant in a labelled microcentrifuge tube with 1.5 ml of 4% PFA and incubate O/N at 4 °C. Rinse the explants twice with 1x PBS.
2. Dehydrate explants through an alcohol series (70% ethanol: 3 times 30 min each; 90% ethanol: 2 times 30 min each; 100% ethanol: 3 times 30 min each; then xylene: 3 times 20 min each). Perform all steps at RT in a fume hood.
3. Imbed tissue explants in paraffin at 58 °C in an oven. Prepare 5 µm thick tissue sections using a rotary microtome.
4. Float the sections in a 56 °C water bath, and then mount the sections onto labeled histological slides. Place the slides in manual staining racks and dry at 65 °C for 1 hr. Allow the slides to cool at RT.
5. Dip the racks in 4 consecutive stain dishes containing xylene for 10 min each to remove paraffin. Dip the racks in an ethanol series to remove xylene: 100%, then 95%, then 80%, then 70%, then 50% ethanol (5 min for every step). Rinse the racks with tap water for 5 min to remove ethanol.

3.3) Hematoxylin and Eosin (H&E) Staining

1. Continue working with the fixed tissue sections; place the rack into a staining dish filled with Mayer's Hematoxylin for 15 min. Rinse the rack with tap water to remove Hematoxylin for 20 min.
2. Place in distilled water for 30 sec. Place in 95% ethanol for 30 sec. Place in Eosin Y solution staining dish for 1 min. Dehydrate through 2 changes of 95% ethanol, 100% ethanol, and xylene for 2 min each.
3. Perform a quick check under a microscope to ensure that excess eosin is removed. Place 2 to 3 drops of Mounting Medium (Fisher SP15-100) onto each slide, then cover with a cover glass.

3.4) In Situ Cell Death Detection: TUNEL Assay

1. Before beginning, prepare proteinase K working solution: 20 µg/ml in 10 mM Tris/HCl, pH 7.4.
2. Repeat steps 1 to 5 of section 3.2 (Explant fixation and tissue section deparaffinization). Rinse the slides with deionized H₂O.
3. Immerse the slides with 1x PBS for 10 min. Drain the excess PBS. Incubate tissue sections for 30 min at RT with proteinase K working solution. Rinse slides twice with 1x PBS.
4. Perform the TUNEL assay as described in the Instruction Manual of cell death detection kit. Mount using mounting medium, and coverslip manually with glass coverslips.
5. Analyze samples under a light microscope. Use Image Pro 4.5 to analyze the apoptotic cells in brown color.

Representative Results

LMPs were characterized with annexin V staining¹⁰ by fluorescence-activated cell sorting (FACS) analysis and gated using 1 µm beads in which 97% of MPs (≤1 µm) were annexin-V-Cy5 positive (**Figure 1A and 1B**). Typically, about 2.5 mg of LMPs were obtained following this protocol. Bronchial tissue explants from C57BL/6 mice were subjected to vehicle and LMPs treatment. Histopathological analysis of bronchial sections revealed the effect of LMPs on the structural integrity of the bronchial epithelium. In control explants, the bronchial epithelium was largely undamaged (**Figure 2A**); however, in LMPs-treated explants, the superficial epithelial cell layer was damaged or lost, and there were significant decreases in epithelial cell height and density (**Figure 2B**). TUNEL-positive staining (a marker of apoptosis) was more pronounced in LMPs-treated bronchial epithelium compared to control (**Figure 3**).

Figure 1. Flow cytometry analysis of MPs derived from CEM T cell line. (A) Determination of forward (FSC) and side scatter (SSC) characteristics with 1 μ m beads used to gate MPs. **(B)** Events in the MPs gate were further assessed for labeling with annexin V-Cy5 to distinguish true events from electronic noise, thereby increasing the specificity of MPs detection. [Please click here to view a larger version of this figure.](#)

a

b

Figure 2. LMPs-induced bronchial epithelial layer damage. Representative histopathological images of bronchial epithelium of explants treated with (A) control or (B) LMPs (40 μ g/ml for 24 hr). Explant sections were stained with Hematoxylin and Eosin. Black arrows point to the epithelial layer. Patchy loss of the superficial and basal cell layers is evident in sections of LMPs-treated bronchial explants (B). Magnification 400X, bar = 20 μ m. [Please click here to view a larger version of this figure.](#)

Figure 3. LMPs-induced bronchial epithelial cell apoptosis. The apoptotic cells in the epithelial layer of segmental bronchi were detected by TUNEL assay; positively stained cells are depicted in brown. Representative images of bronchial epithelial cells in control (**A**) and LMPs-treated groups (**B**) are shown. Black arrows point to the epithelial layer. Magnification 200X (upper panel) and 400X (bottom panel), bar = 20 μ M. [Please click here to view a larger version of this figure.](#)

Discussion

MPs are active mediators of intercellular cross talk and their study is promising in many areas of science.¹¹ This study presented a detailed protocol for *in vitro* large-scale generation of LMPs derived from an apoptotic T cell line. These MPs express a large repertoire of lymphocyte molecules and are biologically implicated in the regulation of cellular and tissue homeostasis. However, LMPs derived from different sources may be biologically different.^{4,9,12,13}

LMPs display varied properties depending on the stimuli used to generate them *in vitro* and the cell from which they are derived. As such, extrapolation of data obtained *in vitro* using LMPs derived from immortalized cell lines to data from LMPs generated *in vivo* should be performed with caution.

This protocol describes several centrifugation steps required for the isolation of MPs; therefore, careful manipulation is needed to minimize the loss of MPs during this process. Snap freezing of isolated LMPs at -70 °C is recommended; we have observed that the bioactivities of LMPs under these conditions can be preserved for up to 2 years (unpublished data).

To date, flow cytometry is considered the “gold standard” for MPs analysis. Polychromatic flow cytometric analysis is used to determine subpopulations of MPs from different cellular origins. Nevertheless, the current commercially available flow cytometers are limited in their ability to analyze smaller-sized MPs populations (less than 300 nm) and to distinguish between cellular debris and MPs. In addition, FACS analysis (cytofluorimetric analysis) may be an alternative method to TUNEL assay to count apoptotic cells for statistical analysis.

Here, we show that bronchial explants cultured *ex vivo* can provide a good source of airway epithelial cells for pharmacology and toxicology screening. Because these bronchial explants resemble their original physiologic environments, they may be useful for determining signalling pathways of certain bronchial or lung diseases. However, use only bronchial explants will not be able to confirm the apoptotic effect of LMPs on epithelial cells is resulted from direct or/and from secondary to the activation of resident immune cells. To investigate the direct effect of LMPs, the primary airway epithelial cells will be appropriate for this purpose.

Disclosures

The authors have nothing to disclose.

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