Microparticles/Exosomes: Isolation and TEM Analysis

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Introduction

Microparticles and exosomes are small vesicular fragments of cell membrane which are released from activated and apoptotic cells. Microparticles (MPs) range in size from 0.5-1.5 μm, and exosomes are 0.5 μm and under (4). For the purposes of this article we will refer to both categories as microparticles. They differ from apoptotic bodies based on their smaller size, intact structure, and lack of degraded nuclear material (11). MPs have been shown to be released from a variety of cell types including platelets, endothelium, vascular smooth muscle cells, dendritic cells, and tumor cells (9). Jimenez (5) and others have shown that based on the stimulus and cell type the MPs released are both quantitatively and phenotypically distinct. More recent data have shown the proteomics of MPs released from human umbilical vein endothelial cells differ dependent on whether they are stimulated with PAI or TNF-α (10).

MPs are rapidly becoming recognized as both a biomarker of disease and health, as well as representing a new signaling paradigm in the vascular system. For these reasons it is necessary to develop methodologies by which to consistently and specifically isolate MPs and examine their contents and structure. Therefore, we have developed a method to isolate and visualize, by transmission electron microscopy (TEM), purified populations of MPs from pulmonary endothelium and breast cancer cells.

Materials and Methods

Isolation of Microparticles

Pulmonary endothelial cells and MDA-MB-231 human breast cancer cells were grown to confluence on 100 mm dishes in DMEM/10%FBS/5%Pen-strep. All media was collected and centrifuged at approximately 100 × g (clinical centrifuge) to remove dead cells and cellular debris. The supernatant was then ultracentrifuged (Beckman L8-70M, rotor TY50, Beckman Coulter, Fullerton, CA) at 100,000 × g for 45 minutes at 4°C. The resulting supernatant was discarded or alternatively used as a depleted control for functional experiments. The MP pellet was re-suspended in serum free media.

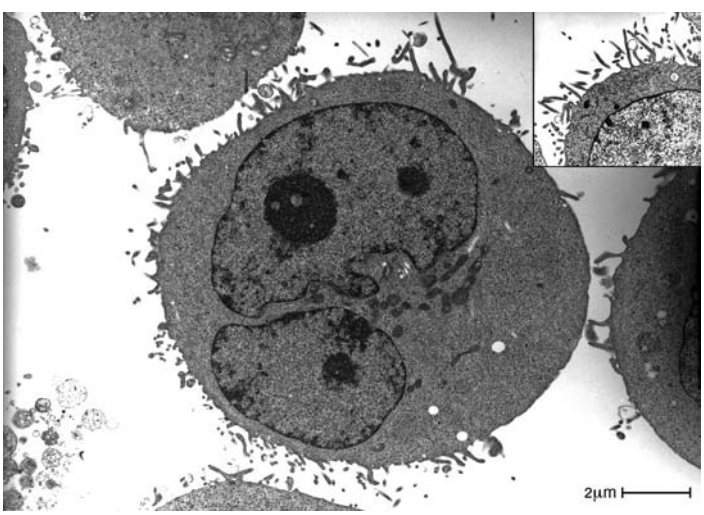

Figure 1. MDA-MB-231 human breast cancer cell in suspension. Note the surface projections, which are finger-like (inset). Compare this cell to the one in Figure 2, which is activated and producing MPs.

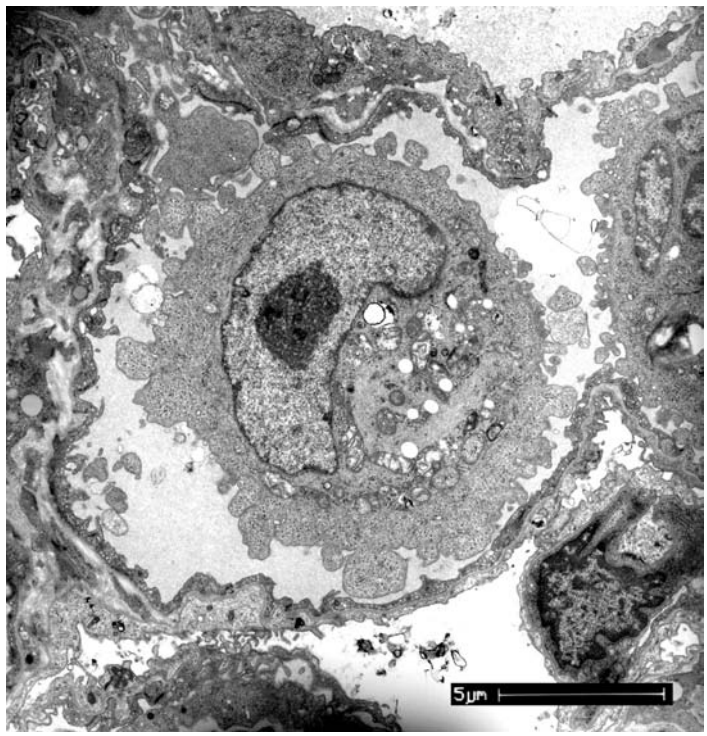

Figure 2. This transmission electron micrograph shows a MDA-MB-231 human breast cancer cell in a distal small vessel in the lung (rat). Note the rounded projections coming off of the surface of the tumor cell. Some of these protrusions ultimately form MPs. Also in the vessel, you can see separate free-floating MPs that could carry the components in the MP to distant areas of the body.

Flow Cytometry

MPs were suspended in 1 mL of either serum free media or PBS and subjected to flow cytometry on a FACS Vantage SE with Diva option by Becton Dickinson (San Jose, CA). For FACS size determination, the flow cytometer was calibrated before each run with 1 μm and/or 0.5 μm Fluoresbrite plain microspheres calibration beads in PBS (Polysciences Incorporated, Warrington, PA). Included in each tube for FACS analysis were 50 μL CountBright Beads (7 μm diameter, Molecular Probes, Eugene, OR).

Transmission Electron Microscopy

MPs were pipetted onto Nunc 10 mm 0.4 micron polycarbonate membranes (Electron Microscopy Sciences, Hatfield, PA) in sterile plates and placed in a humidified incubator for 48 hours. The media was then gently removed and replaced, on both the top and bottom of the filter, with the fixative 3% glutaraldehyde in cacodylate buffer. After cutting out the filters, the specimens were washed in cacodylate buffer, and then post-fixed in osmium tetroxide for 30 min. MPs were rinsed in buffer and then dehydrated in a graded alcohol series. Specimens were embedded in

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O L Y M P U S S O F T I M A G I N G S O L U T I O N S

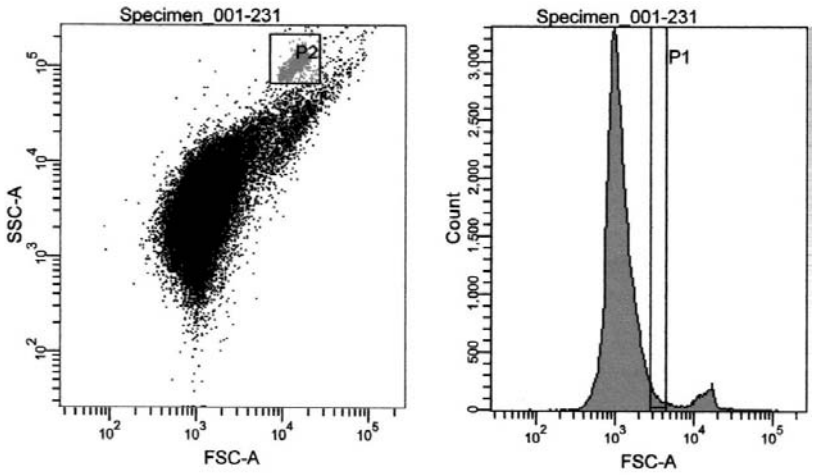

Figure 3. Flow cytometry analysis of microparticles from breast cancer cells (MDA-MB-231). P1 represents the population gating on calibration beads of 1 µm diameter. P2 represents the population gating on 7 µm CountBright counting beads. MPs are defined as the population under 1 µm.

PolyBed 812 (Polysciences, Inc., Warrington, PA). Thin sections (80 nm) were cut with a diamond knife and stained with uranyl acetate and lead citrate. Specimens were viewed and photographed in a Philips CM100 transmission electron microscope (FEI Company, Hillsboro, OR).

Cells in culture were fixed in 3% glutaraldehyde in cacodylate buffer, and processed similar to the isolated MPs described above. Tissue (lungs) were fixed in 3% glutaraldehyde in cacodylate buffer by immersion and then processed similar to that described above.

Results/Discussion

Unstimulated cells have their own characteristic surface contours. Some cells, such as MDA-MB-231 human breast cancer cells, have relatively numerous small projections under baseline conditions (Figure 1). Endothelial cells have fewer projections than tumor cells (7). When cells are stimulated, areas of their surfaces change to bulbous projections that appear to be released in the form of MPs. This occurs in tumor cells (Figure 2) and endothelial cells (6). This phenomenon results in the release of MPs of plasmalemmal or endocytic origin, dependent on the stimulus, which contain proteins, mRNA, or microRNA. Release of MPs from cells associated with the circulation, such as the endothelium or metastatic cancer, allow the particles to continue through the circulation with the blood flow, and thus carry the MP and its cellular components to distant sites (Figure 2). This communication method is currently being studied in the settings of cancer metastasis and pulmonary diseases such as acute lung injury and pulmonary hypertension (1-3).

Isolating purified MPs is key to further analysis by flow cytometry and experiments designed to determine their physiological or pathological function. Our starting materials are purified cell populations; however, other groups have utilized cell surface markers to identify populations of MPs from vast cellular origins in plasma. In collecting MPs from our culture media we found that it is necessary to begin with at least 4×10^6 endothelial cells, but only 2×10^6 cancer cells, to achieve numbers of MPs necessary for continued physiologic or pathologic studies. As we had suspected, cancer cells release significantly increased numbers of MPs under constitutive cell culture conditions as compared to endothelium.

Factors important for flow cytometry include proper size gating based on known parameters and enumeration of the MP population. We used Fluoresbrite microspheres to calibrate the FACS Vantage SE with Diva option flow cytometer. Included in each sample run were CountBright counting beads of known concentration and 7 µm diameter, which we were able to isolate as an independent population from our MPs. Since MPs are considered to be 1 µm and under, our size gate was based on a 1 µm bead and we considered counting any particle under that gate as an MP (Figure 3).

TEM can be utilized to examine MPs from cells in suspension (Figure 1), cells grown on membranes (6, 8), and cells/tissues *in vivo* (Figure 2). TEM confirms the presence of the isolated MPs (Figure 4) and allows for analysis of the substructure, as well as localization of proteins with quantum dots (6, 8). For TEM analysis of isolated MPs, several factors are important. First, due to their small size, it is necessary to give the MPs a substratum upon which to settle in order to prepare them properly for EM. The Nunc polycarbonate membranes provide a support for the MPs during processing for TEM. Since the quantity and size of the MPs is small, having a pellet of them to process for TEM is impractical. To prepare a sample for TEM the specimen must be visible grossly during changes of liquids, during embedding, and during sectioning. The membrane provides a matrix that can be visualized during the processing of the specimen for TEM and the cutting for ultrastructural examination. Note that the MPs must be allowed to settle on the membrane for at least 24-48 hours for them to attach well enough to survive the processing steps.

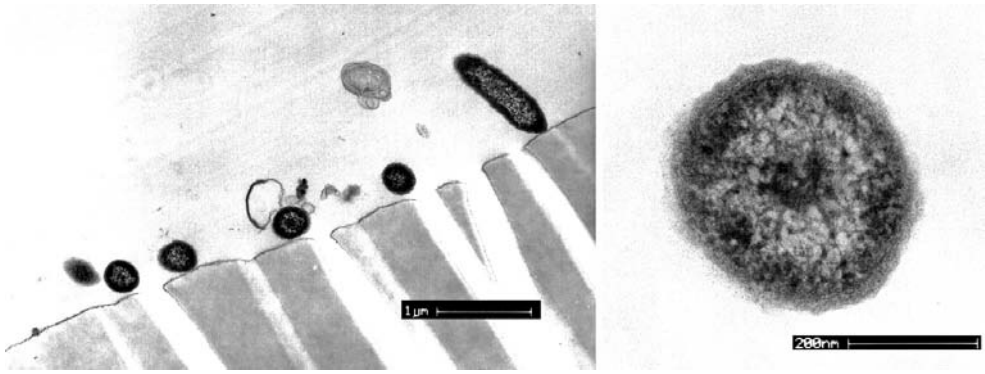

Figure 4 (Left) The transmission electron micrograph shows microparticles from normoxic rat pulmonary microvascular endothelial cells (MVECs) that have settled on the polycarbonate membrane (bottom of the image). Note the preserved ultrastructural detail in this microparticle from MVECs in hypoxia (right).

Ultrastructural detail is well preserved with the method described here. MPs vary from round profiles to oval/cigar-shaped profiles in thin sections (Figure 4a). The internal structure is easily seen in some sections (Figure 4b). Studies are underway to determine the protein components in MPs. Quantum dots have recently been utilized to identify proteins on the MP surfaces (6, 8).

This study has established the methods needed for MP isolation from cells (tumor cells and endothelial cells) in culture and ultrastructural analysis by TEM. Now experiments can be performed to address the proteins and messages in the MPs, and how they function in physiology and pathophysiology. ■

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