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# Isolated microvesicles from peripheral blood and body fluids as observed by scanning electron microscope

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#### ABSTRACT

Microvesicles are sub-micron structures shed from the cell membrane in a final step of the budding process. After being released into the microenvironment they are free to move and carry signaling molecules to distant cells, thereby they represent a communication system within the body. Since all cells shed microvesicles, it can be expected that they will be found in different body fluids. The potential diagnostic value of microvesicles has been suggested, however, a standardized protocol for isolation has not yet been agreed upon. It is unclear what is the content of the isolates and whether the isolated microvesicles were present in vivo or—have they been created within the isolation procedure. To present evidence in this direction, in this work we focus on the visualization of the material obtained by the microvesicle isolation procedure. We present scanning electronic microscope images of microvesicles isolated from blood, ascites, pleural fluid, cerebrospinal fluid, postoperative drainage fluid and chyloid fluid acquired from human and animal patients. Vesicular structures sized from 1 µm downto 50 nm are present in isolates of all considered body fluids, however, the populations differ in size and shape reflecting also the composition of the corresponding sediments. Isolates of microvesicles contain numerous cells which indicates that methods of isolation and determination of the number of microvesicles in the peripheral blood are to be elaborated and improved.

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#### Introduction

Microvesicles (MVs) are membrane-enclosed compartments of the cell interior which are released into surrounding solution in a final stage of the budding process. MVs isolated from blood were for many years considered as an inert platelet dust. It is now evident that they have important roles in vascular haemostasis [1–3], promotion of cancer [4–9] and inflammation [10,11]. As they travel with lymph and blood, they can reach distal cells and interact with them [12]. Following fusion, microvesicle-constituting molecules enter the host cell where they can become functional. This mechanism underlies tumor and infection spreading. Recently, it was found that MVs contain microRNAs (miRNAs)—small regulatory RNAs reflecting the identity of the mother cell, therefore they prove a potential diagnostic and therapeutic system, making a special difference in diseases where

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MVs deriving from platelets contain compounds which catalyze formation of blood clots [15,16], moreover, the area-to-volume ratio of platelet-derived MVs is much larger than that of intact platelets, so that microvesiculation significantly increases the catalytic surface for blood clot formation and is considered as a procoagulant mechanism. The interplay between these processes takes part especially through MV-mediated interaction between platelets, endothelial cells and tumor cells and is reflected in secondary thromboembolic events (e.g. in cancer [17-20] and autoimmune diseases [21]). The abundance of MVs reflects the extent of the membrane pool available for budding and vesiculation, the efficiency of the clearance mechanisms, the properties of the solution surrounding the membrane, and those properties of the membrane that render it more or less likely to bud and vesiculate. Increased levels of circulating MVs were found in patients suffering from cardiovascular disorders [22], cancer [23] and autoimmune diseases [24].

Since their biological role and potential diagnostic and therapeutic values were recognized, efforts are being made to develop relevant

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methods for their gathering, characterization and assessment from peripheral blood [25]. However, it seems that even the simplest taskdetermination of the number of MVs in the sample of peripheral blood is yet a poorly solved problem. Preliminary results show large variation in the number of MVs within the population of persons with no record of a disease [26] indicating that the accuracy, repeatability and robustness of the method for isolation are yet inadequate. Also, flow cytometer scattering showed presence in the samples of isolated material of a rather homogeneous population of MVs of cellular size besides the heterogeneous population of smaller particles [27]. To reveal the origin of the recorded events and therefrom, the identity of the respective recorded fragments, it is of interest to visualize the material isolated from blood as well as of the respective sediments. Besides in blood, MVs were found also in other body fluids, i.e. synovial fluid of inflammed joints [27], pleural fluid [28], ascites [29] and urine [30]. By visualizing material isolated from blood and other body fluids we expected to obtain information on the origin of MVs as well as of the processes taking place in the procedure for isolation. It is the aim of this work to contribute to better understanding of the mechanisms of microvesiculation and detection of MVs. We show electronic microscope images of MVs isolated from different body fluids and respective sediments.

#### Materials and methods

Human body fluids were acquired from patients subject to a written consent. Animal body fluids were obtained from a healthy warmblooded mare and two cat patients subject to a written consent of the owners. Human blood was donated by a patient with locally advanced pancreatic carcinoma (female, 67 years), pleural fluid was obtained from a patient with colon cancer (female, 64 years), and ascites was donated by a patient with perforation of ventriculi and diffuse peritonitis (female, 67 years). Postoperative course was complicated due to multiorgan failure and patient died 3 days after punction. Postoperative drainage fluid was donated by a patient after silicon breast implant replacement (female, 56 years). Cerebrospinal fluid was donated by a patient with subarachnoid hemorrhage (male, 59 years).

Animal blood was obtained from a healthy mare (female, 10 years, 200 kg), chylous fluid was obtained from a domestic cat with chylothorax (female, 15 years), and pleural fluid was obtained from a Persian cat (female, 11 years) with sign of anamnesa dispnoe after ultrasound examination, later diagnosed pulmonary adenocarcinoma and euthanized.

#### Uptake of blood

Human blood was obtained by venipuncture (medial cubital vein) into 2.7 ml vacutubes containing 0.109 M trisodium citrate. Mare blood was obtained by venipuncture (jugular vein) into 2.7 ml vacutubes containing 0.109 M trisodium citrate. Blood uptake was performed according to the Helsinki-Tokyo Declaration following ethical principles.

### Uptake of other body fluids

Body fluids were obtained by punction for therapeutic reasons. The human body fluid samples were collected by a drain tube (about 15–30 cm of length), inserted without needles into pleural cavity, or abdominal cavity, or subarachnoid ventricles (for pleural, ascites and cerebrospinal fluid, respectively). The other end of the tube was inserted into the 2.7 ml vacutube containing 0.109 M trisodium citrate. Due to slowness of drain, cerebrospinal fluid and postoperative drainage fluid



Fig. 1. Material obtained by the protocol for isolation of microvesicles from human peripheral blood plasma (A–D) and sedimented cells from the same sample (E–F).

were first collected into a sterile plastic beaker during several hours and days, respectively, wherefrom vacutubes were filled. In the case of cerebrospinal fluid the insertion of tubes was performed aseptically with general anesthesia.

Chylous and pleural fluids from cats were acquired by insertion of a tube with a needle between shaved and cleaned 7–9 costochondral region, with local anesthesia. The tube was connected to a suction drain system (Mini Redovac, Eickemeyer, Germany). The collected fluid was poured into citrated tubes. During the procedure, oxygen was applied to the cat and afterwards the cat was put into the elevated-level oxygen incubator. Acquired body fluids were collected with standard internationally approved and ethical procedures. The samples were taken from the fluids that were to be discharged.

#### Isolation of microvesicles

Samples were prepared according to the modified protocol proposed by Diamant et al., [25]. Samples were centrifuged for 20 min at 20 °C and 1550 g in vacutubes. Cell sediment was washed with repeated centrifugation in isoosmolar phosphate buffer saline containing anticoagulant (0.109 M trisodium citrate) citrate. To sediment microvesicles, the first centrifugation supernatant was due to centrifuge specifications divided in 1 ml volumes and pipetted into 1.8 ml centrifugation tubes and centrifuged for 1 h at 25,000 and 20 °C. Supernatant was removed and MV pellets were gathered and centrifuged again by the same parameters, until all MV pellets were gathered in one pellet in one centrifugation tube. Supernatant was removed and pellet was washed in 250 ml of PBS–citrate. Final suspension of 250 ml of gathered microvesicles was centrifuged for 30 min at 20 °C and 17,570 g.

#### Preparation of samples for scanning electron microscopy (SEM)

Glutaraldehyde (GA), dissolved in isoosmolar phosphate buffer saline with citrate, was added to the sample to obtain final concentration of 1%. Samples were incubated in GA for 1 h at room temperature (23 °C) and saved at 4 °C until use. Unbound GA was removed from the samples by washing the supernatant 4 times in time intervals of 30 min. OsO<sub>4</sub>, dissolved in isoosmolar phosphate buffer saline with citrate was added to the sample to obtain final concentration of 1%. Samples were incubated for 1 h at room temperature (23 °C). Unbound OsO<sub>4</sub> was removed by washing. To dry the samples, water was exchanged by acetone mixed with citrated phosphate buffer saline. To avoid osmotic shock, acetone concentration was upgraded (50%, 60%, and 90%). The final step was performed in 100% acetone 1 h with two exchanges of acetone. Samples were dried with liquid CO<sub>2</sub>. Dried samples were sputtered with gold to be observed by a Cambridge Instruments S360 scanning electron microscope.

#### Results

Figs. 1A–D show material obtained by the protocol for isolation of microvesicles from human peripheral blood plasma. The sample was rich with microvesicles (C, D), however, numerous platelets and leukocytes can be found in the isolate (B). Figs. 1E and F show sedimented cells from the same sample from which the plasma was obtained by centrifugation. As expected, erythrocytes prevail (E), however, a closer look reveals that many microvesicles are also present in the sample (F). The non-discocytic shape of erythrocytes from blood (F) is a consequence of fixation of erythrocytes while squeezed in the pellet.



Fig. 2. Sedimented cells obtained from chylous fluid of a cat (A, B) and microvesicles isolated from the same sample (C) and from pleural fluid of a cat (D, E). The sediment of the chylous fluid contained mostly leukocytes (A, B).

Fig. 2 shows microvesicles obtained by the protocol for isolation from chylous fluid of a cat, sedimented cells from the same sample (A, B) and microvesicles obtained by the protocol for isolation from pleural fluid of a cat (D,E). The sediment of the chylous fluid consisted mostly of activated leukocytes (A, B) while the isolate from the supernatant was rich in microvesicles indicating that these microvesicles derive from leukocytes. The isolate of the cat pleural liquid contained vesicular structures with low volume-to-area ratio (e.g. stomatocytic shapes).

Fig. 3 shows a sediment and microvesicles isolated from the supernatant of the sample from postoperative drainage fluid in human. Blood cells (A–C: erythrocytes and B, C: leukocytes) and microvesicles (A) are present in the sediment while the supernatant was rich in microvesicles (C–F).

Fig. 4A shows erythrocytes in the sediment of the sample from human cerebrospinal fluid while Fig. 4B shows MVs isolated from the same sample. Figs. 4B and C show MVs isolated from human ascites. Figs. 4E and F show MVs isolated from human pleural fluid. Populations of MVs isolated from cerebrospinal fluid and from ascites seem similar while MVs isolated from pleural fluid are on the average smaller and of more uniform size.

Fig. 5 shows material isolated from peripheral blood of a mare. Numerous activated platelets and globular and tubular microvesicles were found.

## Discussion

In our study we were interested in structural examination and comparison of MVs obtained from different body fluids and also in errors that might occur in the process of their isolation and assessment. We present images of MVs isolated from different human and animal body fluids. As expected, MVs were found in all body fluids. Fig. 1B shows that numerous activated platelets are still present in the sample prepared according to the protocol for isolation of MVs. The sediment shows mainly erythrocytes, however, MVs are also present in the sediment (Fig. 1E). As we have carefully followed the protocol for isolation, it is indicated that the methods for isolation need to be further elaborated in order to yield clinically relevant data. The presence of activated platelets in the sample implies that the isolated MVs consist of two pools: the pool of native MVs which are present in blood at the uptake and the pool of MVs which are shed into the sample after the blood uptake, during the whole isolation procedure. Also, it is possible that some of MVs are dragged into the sediment by blood cells.

Also the samples of the wound drainage and of the cerebrospinal fluid consist mostly of blood cells in the sediment and MVs in the supernatant. Figs. 3A–C show erythrocytes and leukocytes, while Fig. 4A shows a homogeneous population of erythrocytes while the isolated MVs (Figs. 3D–F and 4B) are probably of the platelet origin although we did not observe platelets in the obtained images (Figs. 3 and 4). As is evident from our results that the platelets which underwent the isolation procedure are activated (Figs. 1B and 5A), are therefore prone to shed vesicles and ultimately disintegrate exposing into the sample also exosomes (Fig. 5B). Presence of cells in the samples obtained from peripheral blood by the isolation procedure as described in Materials and methods is revealed also in flow cytometry. A rather homogeneous population of particles sized below 10 µm is detected (not shown), which is in agreement with the observed presence of erythrocytes in the samples.

The size of MVs from the samples of cat chylous fluid is rather large (Fig. 2C). As the sediment reveals predominantly leukocytes it is



**Fig. 3.** Sedimented cells (A–C) and isolated microvesicles (D–F) from a human postoperative drainage fluid. The sample was abundant in erythrocytes (A) while leukocytes could be found (B, C). Numerous microvesicles were found in the supernatant (D–F) as well as in the sediment (A).

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Fig. 4. Sedimented erythrocytes from human cerebrospinal fluid (A) and microvesicles isolated from this sample (B). Microvesicles isolated from ascites of a human patient with perforation of ventriculi and diffuse peritonitis (C, D) and from pleural fluid of a human patient with colon cancer (E, F).

indicated that MVs originate from these cells. In pleural fluid however, MVs are heterogeneous in size and shape. In this particular sample, stomatocytic shapes with low volume-to-area ratio can be observed (Figs. 2D and E).

Population of isolated MVs from human blood, ascites, cerebrospinal fluid and postoperational drainage seem similar in shape and size while the population of MVs from human pleural fluid is more uniform in size while MVs are smaller. Small MVs with uniform size were observed in detergent-induced erythrocyte microvesiculation [31]. On the other hand, pleural fluid MVs have a grain-like appearance. The question arises whether they are actually membraneous microvesicles or are they some other form of microparticles. Additional methods such as biochemical determination of their constituents are necessary to give further insight.

The observed structures in the samples obtained by isolation of MVs are spherical, discoidal, elongated cylindrical and starfish-like. Rounded globular endings are formed as to keep the elastic energy of

the membrane as low as possible. Such shapes have been theoretically determined by the minimization of the free energy of the membrane [32], so it could be claimed that the observed structures correspond to membrane-enclosed fragments of cytoplasm which were formed in a process of increasing spontaneous curvature and/ or an increasing curvature deviator of the membrane [32]. As the curvature of the membrane reaches certain value, a narrow neck is formed which connects the bud with the mother membrane. Due to thinness of the neck-i.e. its small area, the formed bud may be pinched off by shear or other local forces to become a MV which retains the shape of the bud as a consequence of volume and area preservation. Smooth shape of the MV surface can be ascribed mainly to the physical properties of the phospholipid bilayer. In contrast, the observed tubular structures which exhibit sharp edges could not correspond to phospholipid bilayer membrane and are most probably artifacts produced in the process of sample preparation (glass filaments).



Fig. 5. Material isolated from peripheral blood of a mare. In the isolate, numerous activated platelets (A) and microvesicles (B) were found.

Preparation of samples for SEM yields certain artifacts. Nanosized grain-like structures can be observed on the globular core of blood, chylothorax and ascites microvesicles (Figs. 1D, 2C, 4D and F). These structures are gold grains formed as a consequence of excessive gold sputtering by the sample preparation for SEM. Bigger gold grains overshadow the otherwise round core shape of smaller microvesicles from human pleural fluid (Fig. 4F). The stomatocytic form of microvesicles isolated from cat pleural fluid (Figs. 2D, E) as well as the holes on MVs isolated from the postoperative drainage (Fig. 3F) are a consequence of improper dehydration by the sample preparation for SEM.

The use of SEM helped us to determine the possible artifacts that occurred during preparation of samples for SEM as well as those that occurred during MV isolation. The latter are also present by the use of a more elegant and widely used flow cytometer which is the contemporary instrument of choice for examining the MVs. The advantage of flow cytometry is that no fixation and other time consuming and artifactproducing procedures are needed, however, the shape and other microscopic structural features of the sample remain obscure. Imaging of the isolated material thus complements methods for assessment of MVs and helps in localizing the population of MVs in scatter diagrams obtained from flow cytometry. After observing numerous cells in the isolates we have adjusted the protocol for determination of MVs from flow cytometer scatter diagrams to exclude the events that are with high probability ascribed to residual cells in the samples.

We find a reliable and accurate method for the isolation of MVs a crucial step in the proposed studies, especially in clinical studies where populations are compared quantitatively. A thorough analysis of all steps involved in the process of isolation of MVs has not yet been presented. It is therefore prerequisite to improve the existing protocols for isolation of blood MVs in order to create a sound basis for clinical studies. The existing method is sensitive to many parameters in the process such as the blood uptake, the kind and the volume of the anticoagulant in the tubes for blood collection, centrifugation speed and temperature, the fraction of blood plasma taken after separation of the cells from plasma, measurement with flow cytometer and time intervals between protocol steps [27]. In isolation procedure, the incontinent shedding of cells as well as the interaction of membranes with molecules and surfaces in the process of isolation presents a yet unsolved problem. Platelets which are stored for therapeutical purposes were found to vesiculate and are usable only for a certain time interval as they are easily activated in ex-vivo conditions and consequently shed vesicles [33]. It was found that 80-90% of microvesicles obtained from blood are of platelet origin [25]. The overwhelming amount of platelet microparticles which is shed after taking the blood, especially with temperature or shear-activated platelets [34] is therefore detected in the isolated material. Accordingly, lack of platelets in cell sediments might suggest that in the course of body fluid collection and microvesicle isolation a large amount of platelets has vesiculated.

MVs are interesting and a promising field of research due to their suggested roles in various diseases and their potential diagnostical and therapeutical values. Additional effort should be put forward for improvement of the protocol for microvesicle isolation and observation.

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