Les vésicules extracellulaires, potentiels biomarqueurs du cancer de l’ovaire

Alain Brisson

University of Bordeaux, CNRS-CBMN, IPB, Pessac, France
Cells release membrane vesicles in the extracellular milieu upon activation or apoptosis.

- Microvesicles, Microparticles, Exosomes, …
  - Extracellular vesicles (EVs)
  - EVs are found in plasma, urine, synovial liquid, cerebrospinal fluid, ascites fluid, …, in cell culture supernatants.
  - EVs present surface receptors and contain elements (RNAs, miRNAs ..) allowing, in principle, to identify their cell/tissue of origin.
Mechanisms of formation of extracellular vesicles

**Microparticles** (100 nm - 1 µm) form at the cell plasma membrane following cell activation:
- entrance of Ca$^{2+}$ & increase of [Ca$^{2+}$]$_i$
- Ca$^{2+}$-dependent modulation of enzymes
- loss of membrane phospholipid asymmetry
- exposure of phosphatidylserine (PS) on the outer membrane leaflet
- blebbing and shedding of microparticles

**Exosomes** (50 - 100 nm)
- form in multi-vesicular bodies (MVBs)
- are released in the extracellular milieu after fusion of MVBs with the cell plasma membrane
- rich in tetraspanins (CD63, CD81, CD9)
Extracellular vesicles: a plethora of properties & functions
- EVs participate in coagulation, inflammation, inter-cellular communication
- EVs are elevated in many diseases
- EVs promote tissue repair
- EVs contribute to tumor progression,
- etc, …

Extracellular vesicles: potential bio-medical applications
- disease biomarkers – concept of liquid biopsy
- therapeutic agents
- drug delivery systems
- vaccines
<table>
<thead>
<tr>
<th>Title</th>
<th>Authors</th>
<th>Journal/Publication Details</th>
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<tbody>
<tr>
<td>Malignant extracellular vesicles carrying MMP1 mRNA facilitate peritoneal dissemination in ovarian cancer</td>
<td>Yokoi, Yoshioka, Yamamoto, Ishikawa, Ikeda, Kato, Kiyono, Takeshita, Kajiyama, Kikkawa, Ochiya</td>
<td>Nature Communications 8, 6 March 2017</td>
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</table>
Malignant ascites-derived exosomes of ovarian carcinoma patients contain CD24 and EpCAM


Fig. 4. CD24- and EpCAM-positive exosomes can be isolated from malignant ascites fluid of ovarian carcinoma patients.

« Conclusions:

CD24, an established marker of poor prognosis in ovarian and other carcinomas, is contained in exosomes isolated from ascites fluid of ovarian carcinoma patients.

The epithelial cell-adhesion molecule EpCAM, known to be overexpressed in ovarian carcinomas, is secreted in exosomes"
MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer

Fig. 3. Intensities for specific microRNAs derived from the advanced-staged ovarian tumors (□) and from EpCAM-positive exosomes (■) isolated from the sera of these patients.

miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, and miR-214 have previously been demonstrated to be upregulated markers for ovarian cancer.
Detection of phosphatidylserine-positive exosomes as a diagnostic marker for ovarian malignancies: a proof of concept study
J. Lea … A.J. Schroit, Oncotarget, Advance Publications 2017

Figure 3: FACS analysis for PS and CD63 in plasma. A. and B. confirmed cancer patients, C. and D., healthy individuals
The characterization of EVs faces major challenges

1- Lack of reliable methods of EV detection and quantification
   - EVs are small: ~ 50 nm to 1 µm
   - EV suspensions are heterogeneous

2- Lack of efficient methods of EV isolation
   - Most methods are non specific: ultracentrifugation, filtration, size exclusion chromatography, PEG-induced precipitation

3- Lack of standardized methods of EV preparation
Methods for characterizing EVs

\textbf{Single EV detection methods:}
- Flow cytometry
- Electron microscopy
- NTA (nanoparticle tracking analysis), DLS (dynamic light scattering)
- RPS (resistive pulse sensing)
- AFM (atomic force microscopy)

\textbf{Bulk methods:}
- Western blotting, ELISA,
- "Omic" analysis methods: Mass spectrometry, RT-qPCR

\textbf{Functional assays – e.g. measurement of EV procoagulant activity, …}
- SPR (surface plasmon resonance)
- …
Objectives

1) Image EVs in plasma and body fluids
   - EV morphology, size

2) Identify their cell origin

3) Determine their concentration

4) Isolate EVs

5) Determine their composition
   & Identify an EV signature in a pathological sample
Methods

1- Cryo-Transmission Electron Microscopy combined with receptor-specific gold-labeling

→ EV size, morphology, phenotype

2- Flow cytometry

→ EV concentration
Cryo-TEM allows imaging EVs in *pure, unprocessed* plasma & body fluids.
**Principle of Cryo-Electron Microscopy:**
a sample is quickly frozen by plunging in a cryogen, then is observed at low T in the microscope

1. **Preparation**
   - EM grid + specimen
   - liq. ethane (-180°)
   - N₂ liq.

2. **Observation in the EM**
   - -170°C
   - e⁻ → e⁻ → e⁻
   - Thin (≈100nm) droplets of frozen buffer
   - Carbon film (perforated)
   - EM grid

- cryo-EM is the least invasive EM method
- samples are not dried, not fixed, not stained, not thin-sectioned
- samples are observed in their native hydrated state
In plasma, ~80% of the spherical MVs range in size from 50 to 500 nm.

*Cryo-Transmission Electron Microscopy (cryo-EM) allows imaging EVs in pure, unprocessed plasma*

### Spherical MVs - diameter 50 nm – 1 µm

### Tubular MVs - length: 1-10 µm / width: 40-500 nm

*Platelet-Free Plasma (PFP)*
- blood collected over citrate
- 2 x (2,500g x 15 min, 25°C)

(Lacroix et al. *J. Thrombosis & Haemostasis*. 2012 10,437)

Arraud et al., *J. Thrombosis & Haemostasis*, 2014, 12: 614
Cryo-EM combined with *immuno*-gold labeling allows phenotyping EVs

**Objective**: Identify EVs’ origin

- EVs exposing phosphatidylserine
- EVs derived from erythrocytes
- EVs derived from platelets
- EVs exposing EpCAM
- …

**Tool**: protein-conjugated gold particles

**Antibody**
- CD235a
- CD41
- EpCAM
- CD63
- …

**Annexin-5**
Identification of EVs exposing phosphatidylserine (PS)

"Theory": EVs shed from the cell plasma membrane expose PS on their outer lipid leaflet.


Annexin-5 conjugated gold nanoparticles

PS-containing membrane surface
Identification of PS-exposing EVs with Annexin-5 gold labeling

- Labeling is specific
- A minority of EVs bind Anx5/expose PS in plasma …. in "most/all" body fluids
- Advantages of single-step labeling approach
Phenotyping plasma EV by Immuno-Cryo-EM

Erythrocyte-derived EVs
CD235a-Ab gold particles

Platelet-derived EVs - CD41-Ab gold particles

(cell-specific IgG)
Double-labeling allows identifying the cell origin of PS+ EVs.

**Platelet EVs**
- CD41
- 4-nm Anx5-gold-Np
- 10-nm gold-Np

**Erythrocyte EV**
- CD235a

Scale bars: 100 nm (left) and 50 nm (right).
Methods

2- Flow cytometry

→ EV concentration

Conventional flow cytometry method detects only “the peak of EVs’ iceberg”
An alternative method of flow cytometry - fluorescence triggering - improves the quantification of EVs

~ 50 x more EVs are detected by fluorescence triggering compared to conventional light-scatter triggering

# Catalog of EVs in plasma from healthy donors

<table>
<thead>
<tr>
<th>EV phenotype</th>
<th>Spherical EVs</th>
<th>Tubular EVs</th>
<th>Large fragments</th>
<th>Erythrocyte ghosts</th>
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<tbody>
<tr>
<td>FCM</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Anx5+</td>
<td>30 000 ± 6 200</td>
<td>770 ± 270</td>
<td>60 ± 60</td>
<td></td>
</tr>
<tr>
<td>CD41+</td>
<td>22 000 ± 6 000</td>
<td>350 ± 140</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD235a+</td>
<td>13 300 ± 5 300</td>
<td>1 040 ± 420</td>
<td></td>
<td></td>
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<table>
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<tr>
<th>Combination</th>
<th>Count</th>
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<tbody>
<tr>
<td>Anx5+ / CD41+</td>
<td>10 000</td>
</tr>
<tr>
<td>Anx5+ / CD41-</td>
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<tr>
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<td>1 000</td>
</tr>
<tr>
<td>Anx5+ / CD235a-</td>
<td>35 000</td>
</tr>
<tr>
<td>CD235a+ / Anx5-</td>
<td>10 000</td>
</tr>
</tbody>
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1- Caractériser les Vésicules Extracellulaires présentes dans le liquide d’ascite de cancer ovarien
- EVs exposant la phosphatidylyserine, EpCAM, L1, … autres marqueurs

2- Corréler la composition en VEs de l’ascite et du plasma

3- Les Vésicules Extracellulaires peuvent-elles servir de marqueurs du cancer ovarien ?