PERSPECTIVE

Blood extracellular vesicles (EVs) of central nervous system origin: a window into the brain

Extracellular vesicles (EVs) are heterogeneous nano-sized vesicles of endocytic origin shed into blood and other body fluids such as urine, saliva, seminal fluid, ascites, amniotic liquid, synovial fluid, breast milk and cerebrospinal fluid (CSF) by quite all cell types. EVs actively contribute to intercellular communication as they carry bioactive molecules that are selectively incorporated by the originating cell and are delivered to recipient cells over long and short distances (Simons and Raposo, 2009). EVs can be divided into three main groups according to their size and cellular origin: 1) exosomes (40–120 nm), that have an endocytic origin and are formed by inward budding of the limiting membrane of multivescicular bodies, which fuse with the plasma membrane and release exosomes into the extracellular space; 2) microvesicles (50–1000 nm), budding directly off the plasma membrane; 3) apoptotic bodies (> 1000 nm), which are released during apoptosis. Besides originating via distinct processes, different subtypes of EVs carry different proteins within their membrane and luminal compartments that reflect the phenotype of the tissue of origin.

Several studies demonstrated that EVs originated in brain cells could move from the brain to the systemic circulation and vice-versa, crossing the blood-brain barrier (BBB), for this reason exosomes have been recognized as excellent nano-vehicles for drug delivery into brain (**Figure 1**). The mechanism of EVs efflux remain to be elucidated, but some hypothesis of how EVs could move from the central nervous system (CNS) to systemic circulation, have been postulated as follows: 1) direct translocation into capillaries or draining venules via the BBB; 2) passage through interstitial fluid into CSF and then in the venous system via the arachnoid granulations and 3) transportation into the perinasal lymphatics and then into the venous system (Thompson et al., 2016). The presence of EVs of brain origin in peripheral circulation is supported by several evidences. Thus: 1) glioblastoma specific mutant mRNAs can be observed in peripheral EVs (Chen et al, 2013); 2) astrocyte-derived exosomes can be found in the circulation (Goetzl et al, 2016a); 3) brain EVs can be recovered in peripheral blood of genetically modified animals (Mustapic et al., 2017), just to name a few.

Taken together, these findings raised a great interest on peripheral EVs of brain origin, as these EVs could offer an enormous wealth of information for the understanding of the pathological processes that cause neurodegeneration, and could allow to measure the degree of neurodegeneration in real time. This would offer a solution to the fact that no really efficient biomarkers are available to detect the earliest stages of neurodegeneration in pathologies such as Alzheimer's disease (AD) and Parkinson's disease (PD), that affect an increasing number of people worldwide. There is now consensus that the genesis of AD predates dementia onset by over 20 years (Jack et al., 2013). The opportunity of diagnosing patients in the early stages of the disease would be extremely important for such pathologies, when there is still the possibility to maintain an acceptable cognitive status with both pharmacological and rehabilitative treatments. Nowadays the gold standard biomarker for AD remains magnetic resonance imaging together with positron emission tomography. Numerous CSF biomarkers have been proposed, in this scenario, but these are expensive methodologies and lumbar puncture is an invasive procedure limiting its appeal to both patients and physicians. Furthermore, it should be emphasized that these kind of examinations are rarely performed in the absence of specific clinical symptoms. Future non-invasive diagnostic tests involving the analysis of peripheral EVs could be carried out on a large scale even in absence of clinical symptoms in elderly population; this would have an enormous preventative and therapeutic value.

The other side of the coin of this story is the hypothesized direct involvement of the EVs in the pathogenesis of neurodegenerative disease. All forms of AD appear to be characterized by an overproduction and/or a decreased clearance of β-amyloid peptides and tau protein; a similar situation occurs in PD, where the accumulation of misfolded α-synuclein is toxic to neurons. EVs-mediated transmission of the pathologic forms of such proteins between neurons has been proposed to be responsible for the spread of neurodegeneration in the brain.

The method for the isolation of EVs enriched for neuronal origin from blood was first developed by the group of Fiandaca et al. (2015) and repeatedly applied by various research groups. The method consists in a two-step procedure: an initial isolation of total EVs from plasma or serum samples using a commercial available polymer for high-throughput particle precipitation, followed by immunoprecipitation with a

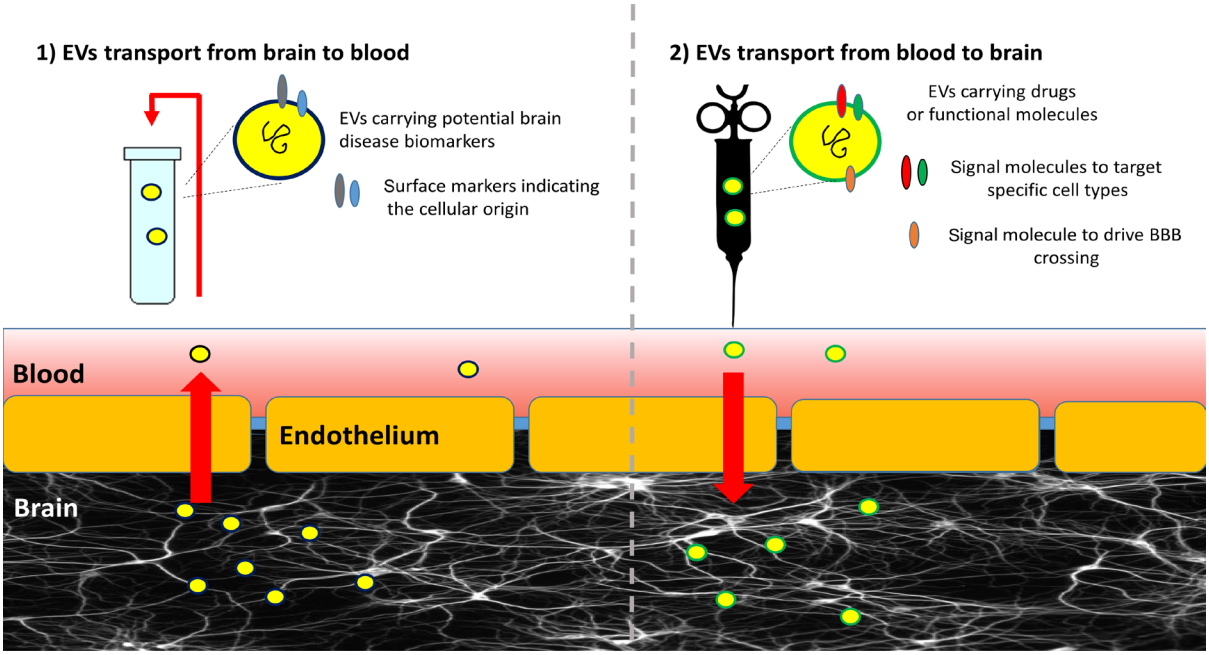

Figure 1 Potential clinical applications exploiting the bidirectional crossing of BBB by EVs.
1) EVs of CNS origin carrying disease specific molecular biomarkers can be isolated from peripheral blood. Their content of proteins and nucleic acids is likely to reflect the pathogenic molecule processes in the cell of origin. 2) Engineered EVs carrying drugs, signal molecules and other functional molecules can be injected in the peripheral circulatory stream and transported via the BBB into the brain to target specific cell types in a variety of CNS conditions. BBB: Blood-brain barrier; CNS: central nervous system; EVs: extravesicles.

biotinylated antibody directed against the neuronal surface marker L1CAM (CD171). L1CAM was selected as target for immunoprecipitation due to its high and relatively specific expression on neurons. The method leads to the isolation EVs of neuronal origin, allowing to evaluate their cargo in terms of proteins, lipids and nucleic acids. The application of this method to a number of different CNS pathologies has led to the generation of interesting results.

Thus Shi et al. (2014) found a significant correlation between plasma exosomal α-synuclein and disease severity, in PD patients. In this case the diagnostic sensitivity and specificity achieved by plasma exosomal α-synuclein were comparable to those determined by CSF α-synuclein. Notably, whereas CSF α-synuclein concentrations are consistently reported to be lower in PD patients compared to controls, plasma exosomal α-synuclein were substantially higher in PD patients (Shi et al., 2014).

Results obtained in AD patients suggested that the concentration of P-S396-tau, P-T181-tau and $A\beta_{1-42}$ in extracts of neurally-derived blood exosomes can predict AD development up to 10 years prior to clinical onset (Fiandaca et al., 2015). More recently, analyses of the concentration of synaptic proteins in neuronal derived EVs extracts of AD patients, frontotemporal dementia patients and controls showed that, whereas synaptophysin, synaptopodin, synaptotagmin-2, and neurogranin were significantly reduced in frontotemporal dementia and AD patients, growth-associated protein 43 and synapsin 1 were reduced in AD patients alone, possibly allowing an early differential diagnosis between these two pathologies (Goetzl et al., 2016b).

Very recently we have shown that the synaptosome-associated protein 25 (SNAP-25) protein can be detected and measured in sera of AD patients and controls (Agliardi et al., 2019). SNAP-25 is a pre-synaptic protein included in the oluble N-ethylmaleimide-sensitive factor attachment protein receptors complex, and it is a marker of synapses integrity in the CNS. We isolated exosomes of neuronal origin (NDEs) from sera of AD patients and controls using the previously described procedure and found that the concentration of SNAP-25 protein is reduced in AD compared to controls. Notably, a correlation between the levels of SNAP-25 carried by NDEs and cognitive status measured by Mini-Mental State Examination score was also detected in AD patients. This was the first report of SNAP-25 measurement in serum and suggest that SNAP-25 carried by NDEs could be an effective and accessible biomarker that reflects synapses integrity in the brain. Previous analyses of protein-unbound SNAP-25 in CSF reported higher levels in AD patients compared to controls (Brinkmalm et al., 2014); autoptic evidence nevertheless showed that SNAP-25 protein is significantly reduced in brain samples of AD patients (Wakabayashi et al., 2014), supporting our results. SNAP-25 in peripheral blood could be contained in NDEs that generate from active synapses in the brain and are actively released by neurons; protein-unbound SNAP-25 in the CSF would result from the continuous leakage of protein from various brain areas into the brain interstitial fluid that clears into the CSF. If this is the case, the measurement of NDE-contained SNAP-25 in serum is a direct reflection of the synaptic loss that characterizes the progression of AD.

The potential clinic application of the analysis of CNS-derived EVs in peripheral blood are many and are promising. Thus, as summarized above, these analyses would lead to the identification and the quantification of disease-specific molecular signatures of CNS disorders transported from brain to blood. Neural derived EVs cargoes of proteins, RNA species as mRNAs, microRNAs and long non-coding RNAs and lipids are likely to reflect core pathogenic intracellular processes in their originating brain cells and could serve as novel easily accessible biomarkers. An extremely promising scenario predicts that EVs carrying signal molecules, drugs or short interfering RNAs could be manufactured and used to actively transport these compounds across the BBB to the brain, to target specific cell types. This would allow to overcome the limitations to brain delivery posed by the BBB itself and offer good biocompatibility, high targeting specificity and low immunogenicity.

The next challenge is the discovery of cellular surface markers that are cell-specific in order to sort EVs subpopulations derived from the different brain cells or brain regions. The recent description of a method resulting in the isolation of neuron-, astrocyte- and oligodendrocyte-derived EVs in plasma by using specific molecular markers (Ohmichi et al., 2019) is extremely important and promising. The characterization of the specific surface molecular signatures of EVs secreted by different brain cells types *in vitro* could allow the identification of biomarkers expressed by CNS-derived EVs in peripheral blood though culture conditions could alter vesicles surface molecules' expression. In conclusion, this new line of research opens the way to a better and more complete clarification of the complex cross-talk between different brain cells, possibly allowing a real understanding of what really happens in the CNS during health and disease.

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