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Cell-derived vesicles in health and disease

A. STURK and R. NIEUWLAND

Human body fluids contain cell-derived vesicles under physiological as well as pathological conditions. These vesicles are released from cells, in particular during conditions of cell activation, stress or programmed cell-death. There are different types of vesicles, best known are microparticles and exosomes (1). Microparticles are relatively large vesicles (diameter 100 nm – 1 µm), which are budded from the plasma membrane. Exosomes are smaller (diameter 30-90 nm), stored as 'intraluminal vesicles' in multi-vesicular bodies, which become released when membranes of multi-vesicular bodies fuse with the plasma membrane (figure 1).

According to the most recent estimates, our body fluids as blood, urine and saliva contain some 10^{11} vesicles/ml (2). The clinical interest in these vesicles is still growing because vesicles promote and initiate blood clotting, promote inflammation, transfer genetic information and bio-molecules between cells thereby affecting disease progression as e.g. in cancer, modulate the immune system, transport parts of viruses and prions, protect cells from waste accumulation, contribute to drug resistance by removing anti-cancer drugs from cancer cells, contain molecules that provide clinical information with regard to the presence of a disease or the development of a disease, and differ in concentration, cellular origin, composition, and function in common diseases as cancer, diabetes and cardiovascular disease (figure 2). In this contribution, we would like to provide an overview of our studies on vesicles in human body fluids.

Cell-derived vesicles and coagulation

The clotting of blood is initiated by a transmembrane protein, Tissue Factor (TF). Under physiological conditions, TF is constitutively expressed exclusively by cells outside the vasculature. Consequently, blood contacts TF only when a blood vessel becomes damaged, such as in a wound area, and then coagulation is initiated. In 1997, we were the first to describe that large numbers of vesicles occur in blood oozing from the wounds in the pericardial cavity of patients undergoing open heart surgery (3). These vesicles initiate coagulation by exposing TF and promote coagulation by exposing negatively charged phospholipids as phosphatidylserine. Moreover, we were the first to demonstrate that these vesicles are capable of inducing TF-mediated thrombus formation *in vivo*, i.e. in a rat venous-stasis model (4). Thus, vesicles from human wound blood can initiate coagulation as well as thrombus formation.

Because both monocytes and endothelial cells express and produce TF upon incubation with endotoxin and release vesicles exposing TF in vitro, we investigated the presence of such vesicles in one of the most devastating infectious diseases known, meningococcal sepsis (5). Plasma from a patient who also developed fulminant disseminated intravascular coagulation contained vesicles from monocytes exposing TF. Reconstitution of these vesicles in plasma revealed that this TF strongly triggers coagulation, thus illustrating that the presence of vesicles exposing TF is associated with development of disseminated intravascular coagulation, at least in some patients.

The story on vesicles and TF is still 'work in progress'. In 2002, we were the first to show that TF can also be present on vesicles in body fluids other than blood. We discovered that the fluid from inflamed joints of arthritic patients contain large numbers of TF-exposing vesicles (6). Because these TF-exposing vesicles strongly triggered coagulation, we hypothesized that the presence of such vesicles, mainly originating from leucocytes, may explain the development of 'rice bodies', the particulate fibrin kernels which can cause considerable pain and irritation. Recently, in 2011, we showed that human

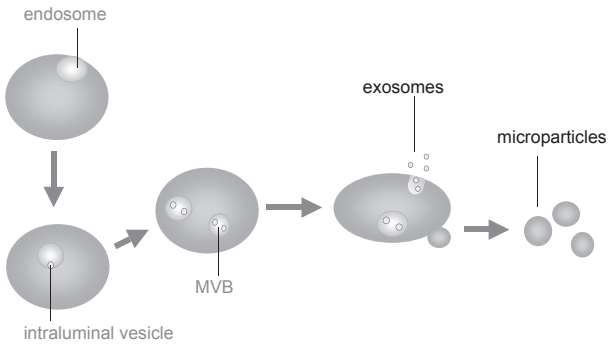

Figure 1. Cell-derived vesicles: microparticles and exosomes. Eukaryotic cells release different types of vesicles into their environment. The two most studied types of vesicles are microparticles and exosomes. The formation of exosomes starts when part of the plasma membrane invaginates, thus forming an endosome. In turn, part of the endosomal membranes invaginates, thereby forming small intraluminal vesicles. When an endosome contains intraluminal vesicles, it is called a multivesicular body (MVB). Finally, when the membrane of a MVB fuses with the plasma membrane, the intraluminal vesicles are secreted and are then called exosomes. Microparticles are formed when a part of the plasma membrane forms a protrusion, a 'bleb', which is released into the environment. *Modified from the Am. J. Reprod. Immunol. 2007; 58: 389-402.*

LEKC, Academic Medical Center, Amsterdam

E-mail: r.nieuwland@amc.nl

saliva also contains TF-exposing vesicles (7). Interestingly, the ability of these vesicles to shorten the clotting time of human blood is comparable to the shortening induced by reptilase, the snake venom that directly converts soluble fibrinogen into insoluble fibrin. Because we hypothesized that TF in human saliva may be an additional source of TF to accelerate coagulation as one of the first steps of wound healing, we added saliva to the pericardial wound blood as the material we started our initial studies with in 1997 and which, as described above, contains substantial levels of TF-exposing vesicles already as source of coagulation-initiating material. Addition of saliva to this wound blood shortens the clotting time of wound blood, confirming our hypothesis that the presence of TF in saliva may indeed be relevant for wound healing.

Vesicles may also play a key role in development of venous thromboembolism (VTE) in cancer patients. VTE is the second cause of death of all hospitalized cancer patients. Blood from most cancer patients contains elevated levels of coagulation activation markers compared to healthy controls. Then what is the cause of this (extrinsic) coagulation activation? In other words, where does TF come from in the blood of cancer patients? Many tumours produce TF and release TF-exposing vesicles that may enter the blood. Tumour-derived vesicles exposing TF are frequently present in blood of cancer patients, strongly suggesting that such vesicles may be (one of) *the* missing link(s) between cancer and the increased risk of VTE.

It is not possible to treat all cancer patients with anti-coagulant therapy *before* development of VTE because of the bleeding risk which outweighs the gain in VTE reduction in the total cancer patient group. Thus, it is necessary to identify those cancer patients who are at high risk of developing VTE to enable anti-coagulant therapy. Previously, we observed that plasma from citrate-anticoagulated blood and depleted from all endogenous vesicles does not clot upon recalcification. Thus, the presence of endogenous vesicles is essential for clotting of plasma, and the clotting time of plasma directly reflects the ability of the vesicles present to initiate (TF) and promote (phosphatidylserine) coagulation unless, of course, there are deficiencies of clotting factors. Therefore, we hypothesized that the presence of TF-exposing vesicles in the blood of cancer patients may make these patients vulnerable to develop VTE.

To study whether the presence of TF-exposing vesicles in blood from cancer patients is associated with an increased risk of developing VTE, blood collected from cancer patients is tested in our 'Fibrin Generation Test' (FGT) (7). In the FGT, vesicle-containing (patient) plasma is recalcified and the clotting time is registered with and without an inhibitory antibody against either TF or activated coagulation factor VII. At present, three Dutch and one Italian hospital contribute to this pilot study, and already more than 200 patients have been included. The results from this pilot study warrant further studies, and the Bouillaud Study, a large multi-centre trial (30 hospitals) involving over 2,500 patients will start in 2012. This study will investigate the efficacy of anti-coagulant therapy *before* development of VTE in cancer patients classified as 'high-risk' by the FGT. In sum, we use the coagulant properties of blood-borne vesicles for personalized therapy.

It should be mentioned that TF can occur in two 'conformations', a coagulant form and a non-coagulant form. Thus, the exposure of TF on circulating vesicles does not necessarily implicate that these vesicles initiate (TF-dependent) coagulation. Indeed, we have shown that in patients with diabetes type II elevated levels of TF-exposing vesicles are present in their circulation, which are associated with components of the metabolic system but do not promote coagulation (8). Because TF has also functions other than coagulation, including angiogenesis and signal transduction, it is temping to speculate that the function(s) of TF, exposed on vesicles, may depend on the physiological or pathological conditions.

Finally, we have shown that blood from healthy subjects contains vesicles, which originate mainly from platelets and erythrocytes (9). Although these vesicles initiate coagulation, there seems to be *no* role for TF under these conditions, but the precise mechanisms how these vesicles initiate coagulation have remained hitherto obscure.

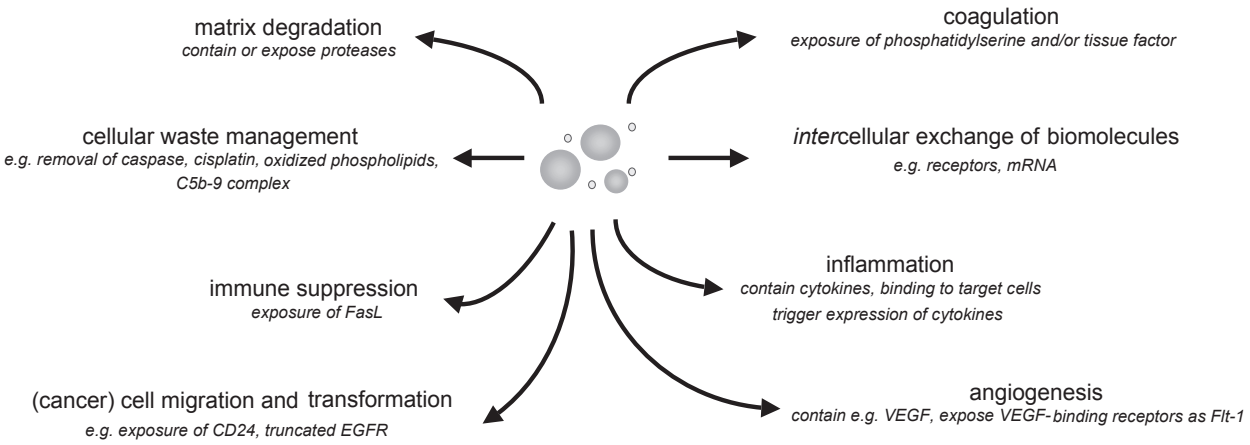

Figure 2. Functions of cell-derived vesicles. The functions of microparticles and exosomes that have gained most attention during the last years have been summarized. C5b-9: terminal complement complex; CD24: Heat Stable Antigen; EGFR: Epithelial Growth Factor Receptor; FasL: Fas Ligand; Flt-1: Vascular Endothelial Growth Factor Receptor 1.

Other functions of cell-derived vesicles

Vesicles are procoagulant, but they also have other functions. In a second line of research we have tried to elucidate *why* cells are releasing vesicles (1). In 2005, we showed that vesicles from healthy, viable human primary endothelial cell cultures contain high levels of caspase 3 and caspase 3 activity (10). Because caspase 3 is one of the main executioner enzymes of apoptosis and the corresponding endothelial cells contained no detectable amount of caspase 3 antigen or caspase 3 activity, we hypothesized that the release of caspase 3-containing vesicles may be a route of 'cellular waste disposal' to enable the cell to avoid apoptosis. Indeed, we observed intracellular accumulation of caspase 3 when the release of vesicles was inhibited, ultimately leading to cell detachment and apoptosis (11). Because vesicles in human blood also contain caspase 3, the role of vesicles as 'cellular dust bins' is likely to occur in vivo and may be of clinical relevance (12). For instance, endothelial cells incubated with clinically relevant concentrations of simvastatin to reduce cholesterol levels remain viable and apparently unaffected (13). Concurrently, a sharp increase is observed of caspase 3 disposal into vesicles, suggesting that cell stress caused by incubation with simvastatin is opposed by the increased release of vesicles. This hypothesis is further confirmed by studies on MCF-7 cells, a human breast cancer cell line. These cells lack caspase 3 due to a large mutation in the procaspase 3 gene. MCF-7 cells do not release any vesicles *in vitro*, but upon transfection with caspase 3-constructs vesicles are released that are 5- to15-fold enriched in caspase 3 activity compared to the cells. Thus, there is sorting of caspase 3 into the vesicles, whereas at the same time caspase 3 itself seems to facilitate its own removal by facilitating the release of vesicles.

Future developments

Detection

The clinical relevance of cell-derived vesicles is clear, but their detection is still a challenge. Since it is now generally accepted that these vesicles behold clinical relevant information, currently a quest is ongoing to develop new and ultrasensitive equipment to detect and quantify single vesicles. The main problem is that most vesicles present in human body fluids have a diameter of less than 100 nm as determined by transmission electron microscopy, and that these vesicles are simply too small to be detected by flow cytometry. Recent estimates are that only 1-2% of all single (large) vesicles are detected using this method (2). At present, we use resistive pulse sensing (RPS) and are able to detect single vesicles with a diameter of 90 nm and larger directly in suspension, e.g. in cell-free body fluids. RPS provides information on the absolute number and size distribution of vesicles present. In addition, in a recent collaboration with the University of Twente, we were able to determine the cellular origin of single vesicles directly in suspension without the necessary antigen-specific antibodies in flow cytometry, by using Raman spectroscopy. In Oxford (UK), a dark field microscope has been combined with the measurement of fluorescence. In this device, called NTA (Nano-Tracking Analysis), single vesicles with a diameter of 50 nm and larger can be measured. Information on absolute concentration and size distribution of vesicles is obtained, as well as information on e.g. the cellular origin of vesicles by using antibodies labelled with a fluorescent probe. In January 2012, we will start pilot experiments measuring the absolute concentration and size distribution of cell-derived vesicles by small-angle X-ray scattering (SAXS) using the four-crystal monochromator of the synchrotron BESSY II in Berlin, which is a collaboration with the Physikalisch-Technische Bundesanstalt.

Clinical relevance

At present there is no doubt that these vesicles provide novel and non-invasive biomarkers, and, similarly, there is also no longer any doubt that these vesicles contribute to mechanisms underlying disease development and progression. For instance, still ongoing studies have shown that plasma from women with ovarian cancer contain tumour-derived exosomes, which can be identified by the presence of distinctive protein members from the claudin family. Detection and identification of such vesicles is likely to be of diagnostic relevance, and may be screening markers in non-symptomatic ovarian cancer patients, especially when compared to insensitive and non-specific markers such as CA-125. Similarly, the prognosis of VTE in cancer patients is a clear example of the clinical relevance these vesicles may behold.

The ability of vesicles to shuttle genetic information or other bio-molecules such as transmembrane proteins (e.g. TF) and receptors or signal transduction elements between cells has received much attention. As such, vesicles may provide a whole new level of intercellular communication, next to hormones. The fact that vesicles can expose cell-type specific adhesion receptors or ligands strongly supports a role for specific interaction and exchange of information between cells via vesicles. Moreover, the presence of intact and functional genetic information such as mRNA and miRNA in vesicles, possibly especially the exosomes, has recently received considerable attention. Again, this information itself can be delivered to target cells, but at the same time may be of diagnostic relevance. For instance, vesicles isolated from blood of pregnant women contain placenta-specific mRNA, which is not detectable in vesicles isolated from blood of non-pregnant women. Such vesicles are likely to contain information whether e.g. preeclampsia is developing in the pregnant woman.

Conclusions

Our studies on the presence and relevance of cell-derived vesicles in human body fluids have contributed to the revival of both the clinical and scientific interest in these vesicles. During the last decade, the vesicles have been upgraded from 'merely an artefact' to an improved understanding of the molecular mechanisms underlying the development of several pathologies. Although our insight into the clinical relevance and understanding of cell-derived vesicles in human body fluids is still in its infants shoes, ongoing research on these vesicles holds many interesting promises for the future.

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máxima
medisch centrum

Schildklierfunctie, zwangerschapsuitkomst en neuropsychologische ontwikkeling van het kind

H.L. VADER en V.J.M. POP

De basis voor deze onderzoekslijn werd gelegd met het promotieonderzoek van Pop naar de relatie tussen postpartale schildklierdysfunctie (postpartum thyroid dysfunction, ptd) en postpartale depressie (postpartum depression, ppd). In 1982 werd voor het eerst in de New England Journal of Medicine het begrip ptd geïntroduceerd, voor Nederland waren tot 1987 nog geen incidentiecijfers voor handen. Evenmin was bekend of schildklierfunctiestoornissen na de bevalling een typisch klachtenpatroon kennen. Er was wel casuïstiek waarin melding gemaakt werd van vrouwen met een ernstige postpartale depressie die bij nader onderzoek een schildklierfunctiestoornis bleken te hebben (1). Studies naar een mogelijke relatie tussen ptd en ppd waren niet eerder uitgevoerd.

Máxima Medisch Centrum, Veldhoven

E-mail: h.vader@mmc.nl

Postpartale schildklierdysfunctie en depressie

In 1987 werd, voor de eerste maal in Nederland, een follow-up studie gestart waarbij een 300-tal zwangeren intensief werd gevolgd. Schildklierparameters (TSH, fT4 en anti-TPO autoantistoffen, toen nog microsomale antistoffen) werden bepaald in de 32e week van de zwangerschap en vanaf de 4e week postpartum iedere 6 weken tot 34 weken postpartum. Bovendien werd het bestaan van depressie gemeten tijdens een diagnostisch interview met behulp van de Research Diagnostic Criteria van Spitzer. De incidentie van ptd in Nederland bedroeg 7,2% (2). Dit betekent dat jaarlijks ongeveer 14.000 vrouwen een ptd doormaken. Hyperfunctie van de schildklier treedt vooral op gedurende de eerste 3-4 maanden na de bevalling, gevolgd door een hypofunctie. Soms kan ook alleen een hypofunctie van de schildklier optreden (3-5). Vooral vrouwen met verhoogde anti-TPO concentraties op 32 weken waren hadden een sterk verhoogd risico voor het ontwikkelen van ptd: RR 20 (95% BI 8,8-45) (6). De (globaal eenjaars)incidentie van ppd bedroeg 21%