Extracellular Vesicles and Vascular Injury: New Insights for Radiation Exposure

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This article reviews our current knowledge about cell-derived extracellular vesicles (EVs), including microparticles and exosomes, and their emergence as mediators of a new important mechanism of cell-to-cell communication. Particular emphasis has been given to the increasing involvement of EVs in the field of radiation-induced vascular injury. Although EVs have been considered for a long time as cell "dust", they in fact precisely reflect the physiological state of the cells. The role of microparticles and exosomes in mediating vascular dysfunction suggests that they may represent novel pathways in short- or long-distance paracrine intercellular signaling in vascular environment. In this article, the mechanisms involved in the biogenesis of microparticles and exosomes, their composition and participation in the pathogenesis of vascular dysfunction are discussed. Furthermore, this article highlights the concept of EVs as potent vectors of biological information and protagonists of an intercellular communication network. Special emphasis is made on EV-mediated microRNA transfer and on the principal consequences of such signal exchange on vascular injury and radiation-induced non-targeted effect. The recent progress in elucidating the biology of EVs has provided new insights for the field of radiation, advancing their use as diagnostic biomarkers or in therapeutic interventions.   © 2016 by Radiation Research Society

INTRODUCTION

Radiation injury of blood vessels was originally identified more than a century ago and continues to be a clinical problem today despite dramatic advances in the field of radiation oncology. Radiation induces endothelial cell (EC) dysfunction, which is characterized by increased permeability, detachment from the underlying basement membrane and apoptosis (*1, 2*). EC dysfunction and apoptosis contribute to postirradiation inflammation and fibrosis. Within vessels, radiation induces a prothrombotic state, which is characterized by platelet aggregation, microthrombus formation and increased adhesion of inflammatory cells to ECs with subsequent diapedesis into the perivascular space (*3*). Structurally, irradiation of the vasculature causes the dose-dependent destruction of blood vessels, which affects the tissue microvasculature in particular (*4*).

The endothelium serves a critical role as a barrier and is the primary sensor of physical and chemical changes in the bloodstream. Endothelial dysfunction is an all-encompassing term for a shift from a normal, healthy endothelium to a stressed/damaged endothelium with a pro-vasoconstriction, pro-coagulation and pro-inflammatory phenotype (*5, 6*). Thus, the recognition of endothelial dysfunction can lead to earlier therapeutic intervention and potentially, reduced vascular damage. More importantly, evaluating circulating biomarkers may reveal mechanisms of endothelial pathology, as well as provide insights on endothelial functional status, while remaining minimally invasive. This article examines extracellular vesicles (EVs) as biomarkers of endothelial dysfunction and discusses their role in vascular homeostasis.

Extracellular vesicles constitute a heterogeneous group of cell-derived vesicles that are enclosed by a lipid bilayer containing various proteins and receptors, which envelopes a diverse array of proteins, nucleic acids, chemicals and structural molecules derived from the cell of origin, the nature of which depends on the cellular source, state and environmental conditions (*7–12*). Nonetheless, three main EV subpopulations have been consistently identified and are classified according to their size and biogenesis (Table 1) (*13–15*). The best studied of these are exosomes (sometimes called nanovesicles), which range in size from 30 to 100 nm. Exosomes are intraluminal vesicles generated by reverse budding of multivesicular bodies (MVBs) within cells before their secretion upon fusion of MVBs with plasma membrane (*16*). A second EV subpopulation consists of microparticles (MPs) or microvesicles (also known as shed vesicles or ectosomes), which range in size from 0.1 to 1 μm. MPs are directly shed from the plasma membrane of cells, arising from regions enriched in lipid rafts and expose phosphatidylserine (PS) in the outer leaflet

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TABLE 1
Classification of Extracellular Vesicles

Vesicle type	Origin	Size	Markers (enriched)	Content
Exosomes	Intraluminal budding of MVBs; fusion of MVBs with plasma membrane	30–100 nm	Tetraspanins (CD63, CD9, CD81), LAMP1, ESCRT components, ALIX, TSG101, MFGE8	mRNA, miRNA, cytoplasmic and membrane proteins
Microparticles	Outward blebbing of cell membrane	0.1–1 μm	PS, surface proteins from cell of origin (integrins, selectins, receptors)	mRNA, miRNA, cytoplasmic and membrane proteins
Apoptic bodies	Blebs released from apoptotic cells	>1 μm	Elevated PS, permeable membrane (PI positive)	DNA, nuclear fragments, cell organelles, proteins, mRNA, miRNA

Notes. MVBs = multivesicular bodies. LAMP1 = lysosomal-associated membrane protein 1; ESCRT = endosomal sorting complex required for transport; ALIX = apoptosis-linked gene 2-interacting protein X; TSG101 = tumor susceptibility gene 101; PS = phosphatidylserine; PI = propidium iodide.

of their membrane (*13*). A third EV subpopulation, is constituted by apoptotic bodies (ABs), which are larger vesicles (1–2 μm) released from apoptotic cells that are rapidly engulfed by phagocytic cells (*9, 14*). ABs are characterized by a permeable membrane, PS exposure and the presence of fragmented nuclear DNA.

All three classes of subcellular vesicles are formed under conditions of endothelial damage, however, the relationship between exosomes and apoptotic bodies and endothelial dysfunction is unclear. EVs have been reported to be part of the disease mechanism in several conditions, such as inflammation and thrombosis, that are reported to be highly involved in the pathogenesis of vascular dysfunction. Finally, given their significant presence in most if not all bodily fluids, which makes them easily and noninvasively accessible, EVs have been investigated as potential biomarkers for many diseases (*17*).

MICROPARTICLES

Microparticle Characterization

The general consensus is that most cell types, including circulating cells and cells present in the vessel wall, are capable of vesiculating and releasing membrane-shed MPs in the extracellular media in response to cell activation or apoptosis. MPs originating from different cell types can be detected in the plasma of healthy subjects, resulting from the active balance between MP generation and clearance. MPs are anuclear fragment of cellular membrane that shed from stressed or damaged cells. With a typical diameter of 0.1–1.0 μm, MPs contain surface proteins and cytoplasmic material of the parent cells. MPs are distinguishable from other subcellular vesicles on the basis of size, mechanism of formation and content (*18*). MPs are typically identified in plasma samples by flow cytometry on the basis of size, the externalization of PS and the presence of specific surface antigens. The labeling of surface antigens allows for identification of the cellular origin of MPs, however, the precise criteria for identification on the basis of surface antigens have yet to be established. In plasma samples, MPs

of endothelial (identified by the surface presence of CD144, CD62E or CD31), platelet (CD41a, CD42b, CD62P), leukocyte (CD45, CD4, CD8, CD14) and erythrocyte (CD235a) origin are present (*19–21*).

Given that MPs are released under conditions of cell stress/damage, it is not surprising that plasma levels of MPs are increased in a wide range of cardiovascular diseases (*15, 22*), cancer (*23*), lung injury (*24*), renal failure (*25, 26*) and decompression sickness (*27*). There is strong evidence to suggest that MPs of endothelial, platelet and leukocyte origin may be reflective of endothelial dysfunction and may in fact contribute to endothelial dysfunction (*13, 28, 29*). The current knowledge about the mechanisms of endothelial vesiculation has been derived mainly from experiments in isolated or cultured ECs, in which their capacity to generate MPs after activation by a variety of stimuli was documented. The data clearly show that endothelial MP shedding can occur independent of endothelial apoptosis (*30*).

Microparticle Stimuli

In addition to the broad structural changes governing MP formation, several signals have been identified that may stimulate or inhibit MP formation. Pro-inflammatory molecules are potent stimuli of microparticle formation from ECs. In this regard, tumor necrosis factor (TNF)-α (*31, 32*), lipopolysaccharide (LPS, in the presence of fatty acids) (*33*), interleukin-1α (*34*) and C-reactive protein (*35, 36*) all promote MP release from ECs. Similarly, the pro-coagulant factors thrombin (*37*) and plasminogen activator inhibitor-1 (*32, 38*) also increase endothelial MP formation. Uremic toxins such as p-cresol (*39*), p-cresyl sulfate (*40*), indoxyl sulfate (*39*) and homocysteine (*41*) are also associated with increased MP formation from ECs. Other stimuli implicated in endothelial MP formation include high glucose (*42*), angiotensin II (*21*), camptothecin (*43*), growth factor deprivation (*44*) and reactive oxygen species (ROS) (*21, 45*). Shear stress has also been associated with endothelial MP formation *in vivo*, although direct effects on isolated ECs have not been reported (*46*). Conversely, statin

treatment (*47*) and NO (*35*) suppress MP production from endothelial cells.

Microparticle Generation

As MP formation begins with the outward budding of the plasma membrane, it is perhaps not surprising that cytoskeletal reorganization is a critical component of MP formation. In this regard, actin filament dynamics appear crucial for MP formation from multiple cell types. Different studies have implicated Rho kinase, an upstream regulator of myosin light-chain kinase and cytoskeletal dynamics, in the formation of MPs from ECs, a process which may involve caspase 2 (*21, 29, 30*). Finally, transglutaminase 2, an enzyme that catalyzes protein cross-linking and governs cytoskeletal reorganization, has recently been implicated in MP release from vascular smooth muscle cells (SMCs) (*48*). Thus, although the precise machinery necessary for MP formation is not fully understood and indeed may differ among various cell populations, cytoskeletal reorganization appears to represent a critical step in MP formation.

A second event implicated in MP formation is the externalization of PS. PS is an aminophospholipid that is found preferentially (if not exclusively) on the inner leaflet of the plasma membrane of healthy cells (*49*). The asymmetric distribution of PS is regulated by three distinct enzymes: flippases, floppases and scramblases. Flippases promote the translocation of PS and phosphatidylethanolamine against their electrochemical gradient towards the inner membrane in an ATP-dependent manner and are constitutively expressed. Floppases, which include members of the ATP-binding cassette (ABC) transporter family, catalyze the transport of PS to the outer membrane in an ATP-dependent fashion. Finally, scramblases are ATP independent and facilitate movement of PS between both membrane leaflets and include TMEM16F (transmembrane protein 16F) (*49, 50*). The majority of studies examining MPs report some degree of outer membrane PS exposure, and emerging evidence suggests that this exposure is a key mediator of the formation of MPs. The strongest evidence supporting this can be found in individuals with Scott syndrome, a condition characterized by an impaired ability to externalize PS and impaired coagulation that may result from defects in TMEM16F or the floppase ABCA1 (*50–53*). Individuals with Scott syndrome exhibit reduced MP shedding from platelets (*54*). Although some reports suggest that PS is not externalized in certain MP populations (an observation based on a lack of detectable annexin V binding), it is unclear whether these populations truly lack externalized PS or the level of externalization is simply below the limits of detection (*55*).

Recently, lipid-rich microdomains known as detergent-insoluble glycolipid-enriched complexes or lipid rafts and caveolae, have also been implicated in the formation of endothelial, monocyte and platelet MPs. Biro *et al.* (*56*) first observed a high cholesterol content in platelet MPs relative to the plasma membrane, suggesting an enrichment in lipid rafts. Additionally, perturbation of lipid-rich domains is associated with alterations in MP formation. In this regard, Liu *et al.* (*57*) have shown that cholesterol enrichment in monocytes/macrophages is associated with enhanced MP formation. Similarly, it has been observed that disruption of lipid-rich domains, with methyl-β-cyclodextrin or nystatin, impaired MP formation in ECs (*21*). Moreover, several proteins that localize to lipid rafts/caveolae have been identified in MPs, including CD39, flotillin-2, endothelial NO synthase and caveolin-1 (*21*). Of note, transport mechanisms not involving protein participation, but requiring large, local deformations of the plasma membrane (e.g., endocytosis, exocytosis and membrane fusion events) are also determined by changes in the composition of lipids.

More importantly, ionizing radiation has been shown to alter the concentration or chemical nature of plasma membrane lipids, which impacts plasma membrane function. In addition, radiation affects the functions mediated by transmembrane proteins by altering their expression or by changing the interaction(s) that normally takes place between membrane lipids and proteins, which can dramatically alter the way in which cells associate with each other within tissues and organs. Altered gap junctional associations, receptor/ligand cell-to-cell communication and ion transport all contribute to loss of tissue homeostasis, carcinogenesis and reduced cell viability (*58, 59*). Radiation-induced damage to the plasma membrane results from lipid peroxidation. Lipid peroxidation is initiated by a hydroxyl radical that initiates a self-perpetuating reaction through formation of lipid radicals, resulting in the oxidative deterioration of polyunsaturated lipid molecules. Membrane lipid peroxidation results in increased membrane permeability to small molecules and ions (*58*).

Recently, it has become evident that lipid rafts also play an important part in signal transduction processes (*60*). Many studies support the model in which rafts merge into larger membrane domains on hydrolysis of sphingomyelin (SM) to ceramide (CER) after exposure to various stimuli. Indeed, CER molecules dramatically change the biophysical properties of plasma membrane, which results in spontaneous self association of rafts to larger domains called platforms. Many receptors or stimuli are able to induce the formation of CER-enriched membrane domains or activation of sphingomyelinase(s) (acidic A-SMase and/or neutral N-SMase), or require the expression of SMase for transmission of the specific biological effect (*60*).

Biological Effects

MPs interact with recipient cells, which may be local or considerably distant from the originating cell, through a process entailing ligand/receptor signaling at the recipient cell surface and/or the fusion of vesicle and cell plasma membranes. Mechanisms involving integrin interaction have also been reported for MPs derived from other cell

types (ECs, SMCs and leukocytes). Recently, Burger *et al.* (*21*) showed that direct interaction of heparin-binding EGF-like growth factor/positive MPs with the endothelial EGF receptor causes pro-oxidative and pro-inflammatory responses in ECs *in vitro*. Lipids, in particular externalized PS, are a key determinant of the interaction of membrane vesicles with target cells, although the PS-moiety of endothelial MPs can interact with endothelial PS receptor in an annexin I-dependent manner to prevent endothelial apoptosis (*61*). The biological effects of MPs have been extensively reviewed elsewhere (*22, 62, 63*). Discussed below are some aspects of MP biology related to vascular dysfunction.

Coagulation. In human diseases, the contribution of MPs originating from ECs to the circulating pool of tissue factor (TF)-positive MPs has already been documented in sickle cell anemia (*64*) and sepsis (*65*). Moreover, these MPs induce TF expression of monocytes after binding and thus, participate in the amplification of pro-coagulant cellular responses (*66*). Endothelial MPs (EMPs) harboring PS and TF-dependent pro-coagulant activity are released from cultured ECs, in response to a variety of stimuli including cytokines, complement and LPS (*67–69*). This is consistent with the endothelial expression of TF detected in animal models of endotoxemia and also in sickle cell mice (*70, 71*).

The discovery of fibrinolytic activity harbored by MPs further adds to their contribution in the regulation of hemostatic balance. This fibrinolytic activity is related to the evidence of molecular equipment that identifies MPs as an efficient support for plasmin generation. The plasmin generation capacity of MPs was more extensively studied in EMPs. This property has been initially demonstrated in a model of MPs generated from human microvascular ECs (HMECs) stimulated with TNF-α, in which the urokinase plasminogen activator (uPA/uPAR) system was identified as the major activator (*72*). Finally, tissue (t)-PA was also characterized as the activator that was detectable on the surface of MPs from human primary micro- and macro-vascular ECs and was then further confirmed in MP samples from patients with thrombotic thrombocytopenia purpura and systemic lupus erythematosus (*73*).

Apart from their well-known pro-coagulant activities, platelet-, leukocyte-, endothelial- and cancer-derived MPs harbor a variety of anticoagulant factors, reflecting the pro- and anti-coagulant properties of their parent cells. This equilibrium reflects, at least in part, the coagulable state and could be useful in identifying thrombotic risk (*74*). Another regulatory mechanism by which EMPs and monocyte-derived MPs are thought to counteract thrombin generation is the exposure of the anticoagulant receptors thrombomo-dulin and the endothelial cell protein C receptor (*75, 76*). Platelet-derived MPs have also been found to accelerate factor Va inactivation by activated protein C (*77*).

Ionizing radiation is associated with an increased risk of thrombotic events, with delayed re-endothelialization, fibrin deposition and platelet recruitment as potential causes of postirradiation thrombosis (*78*). In our laboratory, we investigated a cohort of 217 patients from the Epinal accident (France), where patients with prostate adenocarci-noma were overexposed to radiation during radiotherapy (*79*). We demonstrated that MPs isolated from peripheral blood samples could be used as potential biomarkers in association to the severity grade of the complications in this patient population. Furthermore, this study identified the cellular origin of MPs present in chronic radiation enteritis patients and demonstrated that the higher circulating MP level observed in severe-grade patients was due to an increase of MPs derived from platelets, monocytes and ECs (unpublished data). These results are in accordance with an elevation of these subpopulations in acute coronary syndrome, hypertension or atherosclerosis and in all thrombotic diseases occurring in both venous and arterial beds (*46, 80–83*). Our *in vitro* studies confirmed that radiation triggered a dose-dependent release of MPs by platelets. Moreover, it has been shown that radiation induced TF expression and an increase in the pro-coagulability of MPs derived from human peripheral blood mononuclear cells (*78*). These findings may explain a possible mechanism by which radiation enhances blood thrombogenicity (Fig. 1).

Inflammation. There is now emerging evidence from *in vitro* and *in vivo* studies that MPs may play a role in inflammatory conditions, since they display a variety of pro-inflammatory activities. MPs from ECs, platelets and leukocytes can promote adhesion and rolling of leukocytes, contain pro-inflammatory cytokines and trigger the release of MPs from several cell types *in vitro* (*84, 85*). In addition, oxidized phospholipids from endothelial MPs released by oxidative stress may cause monocyte adherence to ECs and neutrophil activation (*85*). A recent study demonstrated also that MPs, isolated from septic shock patients, injected into rats induced the expression of inducible NO synthase, nuclear factor kappa B (NFκB) and cyclooxygenase-2 in the lungs and hearts of these animals (*86*).

Inflammation is one of the major consequences of radiation injury and adhesion molecules are known to play a key role in cellular traffic through vascular endothelium when leukocytes migrate from blood into tissues (*87*). In our laboratory, we observed endothelial activation in a P-selectin-dependent manner related to incubation of MPs isolated from patients with prostate adenocarcinoma who were overexposed to radiation during their radiotherapy (unpublished data). Similarly, our results have shown vascular cell adhesion molecule-1-dependent endothelial activation. Our *in vitro* studies confirmed that radiation triggered a dose-dependent release of MPs by monocytes and ECs. CD31⁺ and CD41⁺ MPs are considered to be valuable surrogate markers for reflecting the extent of EC dysfunction, and CD14⁺ MPs could be considered as pro-inflammatory molecules that enhance vascular inflammation (*88, 89*).
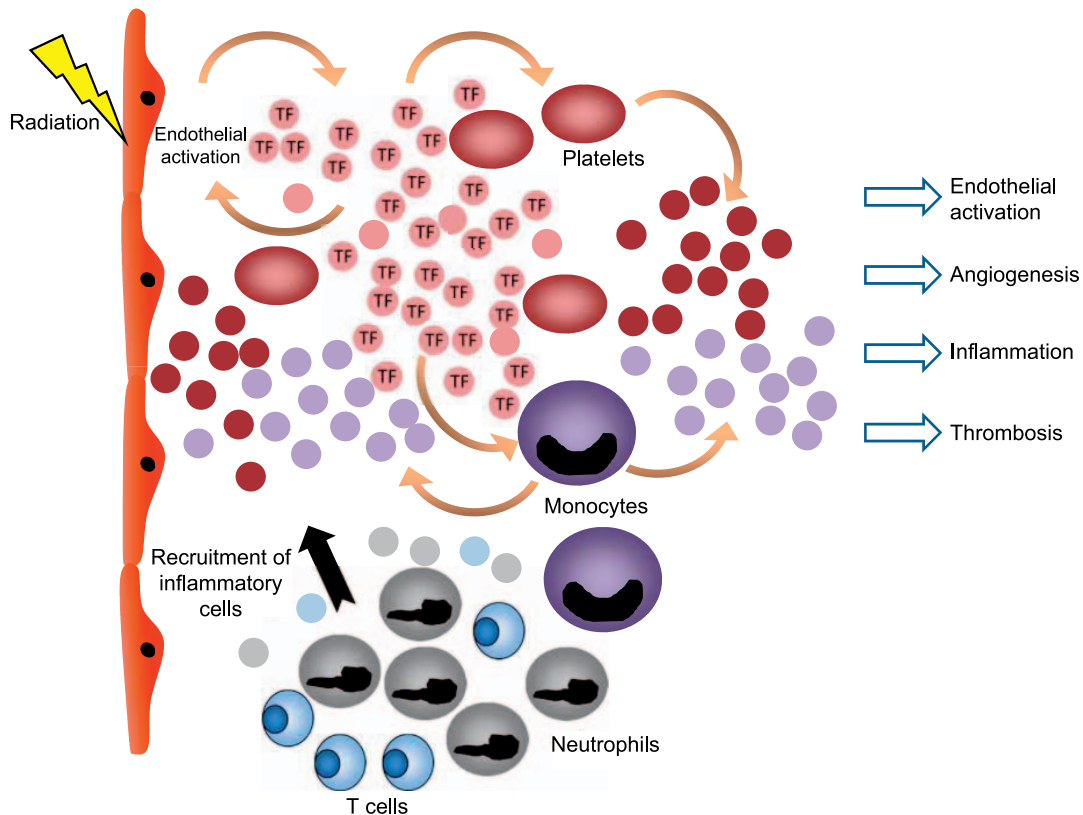
FIG. 1. Schematic representation of the microparticle secretion after radiation-induced endothelial activation. MPs could be considered as signalosomes for several core biological processes. For example, microparticles may activate immune responses. In blood circulation, extracellular vesicles participate in the coagulation cascade by providing a surface for the assembly of clotting factors. Microparticles also take part in stem cell maintenance and plasticity, and they appear to have an essential role in the repair of injured tissue owing to their neoangiogenic, anti-apoptotic and cell proliferation-stimulating characteristics. Given their involvement in disease progression, extracellular vesicles can be considered as targets for therapeutic intervention as well as useful disease biomarkers.

Angiogenesis. Different studies demonstrated that MPs play a part in development, angiogenesis, wound healing and, more generally, tissue remodeling, in the form of positive or negative gradients of information delivered to neighboring cells (*15, 90*). It has been shown that platelet MPs from healthy individuals promote proliferation, migration and tube formation in cultured ECs. The latter effects of MPs are mediated by their lipid components, probably sphingosine 1-phosphate. The ability of platelet MPs to induce angiogenesis is related to the activation of extracellular signal-regulated kinase and phosphoinositide 3-kinase pathways (*91*). Also, MPs of endothelial origin can elicit angiogenesis, but the mechanisms by which they mediate their effects are different from those reported for platelet MPs. Indeed, metalloproteinases harbored by endothelial MPs regulate the focalized proteolytic activity essential for invasion during neovascular structure formation (*92*). Although these effects have been described in *in vitro* systems, one would expect that this effect of EMPs may contribute to neovascularization in *in vivo* situations (*93*). Promotion of angiogenic processes by EMPs may

have both beneficial and deleterious effects. EMPs could be endogenous survival signals responsible for vascular repair in ischemic tissues. However, promotion of angiogenic response may also have deleterious effects in cancer spreading, proliferative diabetic retinopathy (*94*) or atherosclerotic plaque destabilization by promoting intraplaque neovascularization (*95*). These examples suggest that involvement of EMPs in vascular homeostasis is more complex than initially thought and demonstrate that further studies are needed to examine the mechanisms involved (*68*). However, the effects of radiation on the angiogenic process need to be more fully investigated.

EXOSOMES

Exosome Biogenesis and Characterization

Exosomes are a population of vesicles of endocytic origin that are formed as intraluminal vesicles (ILVs) by budding into early endosomes and MVBs. Cargo sorting into exosomes involves the endosomal sorting complex required for transport (ESCRT) and other associated proteins such as

apoptosis-linked gene 2-interacting protein X (ALIX) and tumor susceptibility gene 101 (TSG101). Recently, several ESCRT-independent mechanisms were also described in ILV formation and exosome biogenesis, involving lipids, tetraspanins or heat shock proteins (*7, 96*). Of note, mammalian cells depleted for key ESCRT components still form MVBs (*97*). Therefore, in some cells exosome production requires the lipid CER and nSMase (*98*). Exosomes are secreted after the fusion of MVBs with the cell membrane, which depends on several small Rab GTPases (*99*). Therefore, exosomes differ from MPs or apoptotic bodies that are released from the cell as a result of a direct budding process of the plasma membrane. The ultrastructure of exosomes obtained by transmission electron microscopy appears to be cup shaped, which is likely due to the collapse of these circular molecules as a result of processing and fixation. Indeed, quickly frozen exosomes analyzed by cryo-electron microscopy have a perfectly round shape (*7*). Typically, exosomes have a diameter of 30–100 nm and a density of 1.13–1.19 g/ml and are isolated through sucrose cushion or density gradient by ultracentrifugation at 100,000*g* (*100*).

Exosomes contain cytoplasmic proteins, certain lipid raft-interacting proteins and RNAs, however, owing to their highly regulated biogenesis, exosomes typically accommodate some additional defined components. These include endosome-associated proteins, such as: flotillin; ALIX; TSG101; the tetraspanins CD63, CD9 and CD81; and the lipids sphingomyelin, ceramides and cholesterol (*7*). Of interest, there have been numerous reported on the RNA contents of EVs isolated from cell cultures or body fluids (*101–103*). Although most studies focused on the presence of microRNAs (miRNAs), recent deep sequencing analyses revealed the presence of other small noncoding RNA species, many of which were found to be enriched in EVs relative to cellular RNA, suggesting a selective incorporation of vesicular RNA molecules (*101, 103, 104*). Once secreted from the cell, exosomes can deliver their cargo to adjacent or distant cells, including macrophages, ECs and tumor cells, thereby modifying the target cell's gene expression, signaling and overall function. In most cases, this leads to EV uptake through endocytosis. During this process, EV membrane constituents can be delivered to the recipient cell membrane and EV cargo can enter the recipient cell cytoplasm or nucleus, thereby contributing additional signaling molecules and pathways and potentially resulting in a diverse range of functional consequences in the recipient cell (*9, 105, 106*).

EV-Mediated MicroRNA Transfer and Vascular Homeostasis

EVs transfer functional miRNA into recipient vascular cells. Valadi *et al.* first demonstrated the transfer of miRNA through EVs between mast cells in culture (*101*). EV-mediated miRNA transfer has since been demonstrated in many cellular settings, including the cardiovascular system, where vascular (ECs, SMCs, pericytes), cardiac (cardiomyocytes, fibroblasts) and stem/progenitor cells [mesenchymal stromal cells (MSCs), cardiac progenitor cells, endothelial progenitor cells (EPCs), circulating CD34$^+$ cells] were shown to interact with each other by exchanging biological material through EV production/uptake (*107*). Transfer of functionally active miRNA has been validated by elegant *in vitro* studies showing the uptake of EVs released from macrophages or platelets by ECs in culture (Table 2). Laffont *et al.* demonstrated that miR-223-containing MPs released by platelets were internalized by ECs, where miR-223 regulated the expression of endogenous target genes (*108*). Squadrito *et al.* showed that macrophage-derived exosomes transferred a number of miRNAs in ECs, of which miR-142-3p was shown to regulate reporter gene expression (*109*). EV-mediated transfer of functionally active miRNA was also shown to exert a functional impact on recipient cells and vascular homeostasis. Expression of the miR-143/145 cluster was shown to be induced in ECs in response to shear stress, through Kruppel-like factor-2 transcription factor upregulation. These miRNAs were transferred through EVs to SMCs, where they regulated the expression of several target genes (*110*). The miR143/145 cluster is predominantly expressed in SMCs, where it plays an important role in the regulation of differentiation and proliferation (*111*). Accordingly, *miR-143/145$^{-/-}$* mice display a thinner arterial medial layer and a decreased blood pressure, and they develop spontaneous neointimal lesions in the femoral arteries (*112*). Injection of EVs containing miR-143/145 in *Apoe$^{-/-}$* mice on a high-fat diet resulted in the reduction of plaque size, thus conferring an atheroprotective function to these miRNAs (*110*). Interestingly, it was recently shown that miR-143/145 could be transferred in the opposite direction as well, through the formation of tunneling nanotubes (membrane protrusions), highlighting the importance of these miRNAs in cell-to-cell communication among SMCs and ECs for the regulation of EC function and vascular homeostasis (*113*).

Angiogenic function of EV-derived miR-126. Zernecke *et al.* demonstrated that human umbilical vein EC (HUVEC)-derived ABs were generated during atherosclerosis, and that these ABs were enriched for miR-126 (*114*). AB uptake by recipient ECs led to the miR-126-dependent induction of *CXCL12* expression, through inhibition of miR-126 target gene *RGS16*. It was finally shown that treatment with ABs released from apoptotic HUVECs, or isolated from atherosclerotic plaques from patients undergoing endarterectomy of carotid arteries, reduced plaque size in *Apoe$^{-/-}$* mice on a high-fat diet, through the *CXCL12*-dependent recruitment of progenitor cells for endothelial repair. This effect was abrogated when using ABs obtained from *miR-126$^{-/-}$* mouse ECs compared to *miR-126$^{+/+}$* ECs, suggesting an atheroprotective role for miR-126 (*114*). Jansen *et al.* obtained similar results showing that EVs from apoptotic human coronary artery ECs promoted vascular EC repair by

transferring mir-126 to ECs *in vitro* and *in vivo*, using a re-endothelialization model after electric endothelial denudation of the common carotid artery in mice (*115*). This effect was mediated, at least in part, by inhibition of the miR-126 target gene *SPRED1*, resulting in enhancement of vascular endothelial growth factor (VEGF) signaling (*116, 117*). It is interesting to note that the two aforementioned studies demonstrated a similar atheroprotective role for miR-126-containing EVs released from apoptotic ECs, yet Zernecke *et al.* attributed this function to endothelial ABs, while Jansen *et al.* worked with endothelial MPs. Although these two classes of EVs are produced through distinct mechanisms and display different sizes (*12, 118*), they both expose PS at their surface, which was used for vesicle characterization in these two studies, and the procedures used for their isolation were similar, including removal of cells and cell debris at 800–1,500*g* for 10 min, followed by pelleting of EVs at 16,000–20,000*g* for 20–40 min. It is therefore tempting to speculate that these two studies indeed tested the same EV population, which was likely a mixture of both types of vesicles (*118*). Admittedly, this observation does not preclude the atheroprotective effect of apoptotic EC-derived EVs, though it remains unclear whether it is associated solely with ABs, MPs or both.

It has been well established that endothelial miR-126 governs vascular integrity and angiogenesis by regulating the responses of ECs to VEGF and fibroblast growth factor (FGF). Targeted deletion of miR-126 in mice and its knockdown in zebrafish causes vascular leakage during embryonic development and defective neovascularization after myocardial infarction, due to defects in the proliferation and migration of ECs and angiogenesis (*116, 117*). It is therefore not surprising that EV-mediated transfer of miR-126 to ECs exerts similar pro-angiogenic effects. Thus, it was found that bone marrow-derived CD34$^+$ peripheral blood mononuclear cells secrete EVs with angiogenic activity both *in vitro* and *in vivo*. These EVs were enriched for miR-126, and treatment with anti-miR-126 abrogated the pro-angiogenic effect of CD34$^+$ cell-derived EVs. Reduced miR-126 expression in high-glucose-treated or in type 2 diabetic patient-derived CD34$^+$ cells resulted in altered pro-angiogenic capacity, although it could be rescued by miR-126 mimics (*119, 120*). Of note, EVs released by ECs grown in high-glucose conditions also showed reduced levels of miR-126, and reduced regenerative capacity *in vitro* and *in vivo* (*115*). These observations suggest a role for reduced circulating EVs miR-126 expression in the vascular impairment associated with diabetes mellitus, corroborating miRNA profiling studies showing reduced plasma miR-126 levels in type 2 diabetic patients (*121*). Interestingly, miR-126 was also reported to be transferred in a vesicle-independent manner from ECs to SMCs in a coculture system *in vitro*, where it regulated the expression of target genes *FOXO3*, *BCL2* and *IRS1*, leading to increased proliferation of SMCs (*122*). This effect was abolished when applying laminar shear stress to ECs. The antiatherogenic effect of shear stress, mediated by inhibition of miR-126 secretion, was confirmed *in vivo* using a carotid artery ligation model in which the neointimal lesion formation seen in wild-type animals was attenuated in *miR-126$^{-/-}$* mice. These studies suggest that miR-126 may exert pro- or anti-atherogenic functions, depending on its mode of extracellular transfer and recipient cell type.

Other angiogenesis-related miRNAs. Other EV-transferred miRNAs were shown to play a role in promoting and regulating angiogenesis (Table 2). The human monocytic cell line THP-1 was shown to transfer miR-150 to recipient ECs via EVs, where it decreased expression of its target gene *MYB*, resulting in enhanced cell migration. Interestingly, elevated levels of miR-150 were also found in EVs from plasma of patients with severe atherosclerosis, similarly resulting in decreased c-Myb protein level in recipient ECs and enhanced cell migration (*123*). Exosomes released by the human microvascular endothelial cell line HMEC-1 in culture were shown to stimulate angiogenesis through the transfer of miR-214 in recipient HMEC-1 cells, resulting in reduced expression of target gene *ATM*, leading to repression of cell senescence (*124*). HMEC-1-derived exosomes with reduced miR-214 levels had reduced capacity to stimulate HMEC-1 cell migration and angiogenesis *in vitro* and *ex vivo* (Matrigel® plug assay). In addition, miR-132 was shown to be produced and secreted by saphenous vein-derived pericyte progenitors (SVPs), especially in response to hypoxic culture conditions (*125*). Exposure to SVP conditioned media (CM) led to increased miR-132 levels in HUVECs, accompanied by inhibition of its target gene *p120RASGAP*. Notably, the pro-angiogenic capacity of SVP-CM was blunted upon transfection with anti-miR-132 in SVPs, leading to reduced HUVEC proliferation and tube formation. However, the method of transportation between SVPs and recipient ECs was not investigated in this study. Interestingly, miR-132 inhibition attenuated the effect of SVP transplantation on promoting reparative neovascularization in a mouse model of myocardial infarction (*125*).

Angiogenic potential of miRNAs from cancer-derived EVs. Exosome-mediated miRNA transfer from cancer cells to the niche was recently reported to promote metastasis through vascular remodeling. Zhou *et al.* showed that miR-105 is overexpressed in metastatic breast cancer MDA-MB-231 cells and their exosomes, compared to primary tumor cells (*126*). Exosome-mediated transfer of miR-105 to HMVECs induced EC migration and led to the inhibition of the target gene *TJP1/ZO-1* (tight junction protein 1/zonula occludens-1). MDA-MB-231-derived exosomes induced vascular destruction and increased vascular permeability *in vitro* and *in vivo*, thereby promoting metastasis in the lung and brain. This effect was suppressed through inhibition of miR-105, which restored *ZO-1* expression in vascular ECs and the barrier function of endothelial niche cells. Similarly, Kosaka *et al.* found that exosomes derived from metastatic breast cancer 4T1 cells enhanced tube

TABLE 2
Overview of the miRNAs Transferred by Extracellular Vesicles Discussed in This Article

Producing cells	Species	Vesicle type (as indicated in original publication)	Purification method
Platelets	Human	Microparticles	Centrifugation 20,000g, 90 min
Primary and immortalized BM-derived macrophages	Mouse	Exosomes	Precipitation (Exoquick, primary Mϕ) and ultra 134,000g, 70 min (immortalized Mϕ)
KLF2-transduced HUVECs; mouse lung ECs	Human; mouse	Extracellular vesicles	Centrifugation 20,500g, 60 min
Apoptotic HUVECs	Human	Apoptotic bodies	Centrifugation 16,000g, 20 min
Apoptotic HCAECs	Human	Microparticles	Centrifugation 20,000g, 40 min
CD34$^+$ PBMCs	Human	Extracellular vesicles (MVs and exosomes)	Centrifugation 16,000g, 60 min (MVs) + ultra 120,000g, 60 min (exosomes)
CD34$^+$ PBMCs	Human	Exosomes	Ultra 100,000g, 60 min (sucrose gradient)
THP-1 monocytes	Human	MVs (exosomes)	Ultra 110,000g, 120 min
HMEC-1	Human	Exosomes	Ultra 100,000g, 60 min
Pericytes (SVPs)	Human	*ND* (CM)	Ultrafiltration (50× concentrate)
MDA-MB-231 breast cancer cells	Human	Exosomes	Ultra 110,000g, 110 min
4T1 breast cancer cells	Mouse	Exosomes	Ultra 110,000g, 70 min
Hypoxic K562 leukemia cells	Human	Exosomes	Precipitation (Exoquick)
Hypoxic multiple myeloma cells	Human	Exosomes	Precipitation (Exoquick)
Irradiated MRC-5 fetal lung fibroblasts	Human	Exosomes	Precipitation (Exoquick)

Notes. HUVECs = human umbilical vein endothelial cells (ECs); n.d. = not done; BM = bone marrow; ultra = ultracentrifugation; SMCs = smooth muscle cells; HCAECs = human carotid artery ECs; PBMCs = peripheral blood mononuclear cells; HAECs = human aortic ECs; HMEC/HMVECs = human microvascular ECs; SVPs = saphenous vein-derived pericyte progenitors; CM = conditioned media; RIBE = radiation-induced bystander effect.

formation and migration of HUVECs *in vitro* (*127*). This effect was partially mediated by exosomal transfer of miR-210 from cancer cells to HUVECs, where it regulated reporter gene expression. Expression of miR-210 is regulated by hypoxia-inducible factor (HIF)-1, and has been reported to induce tube formation and migration of HUVECs in response to hypoxia *in vitro* (*128, 129*). miR-210 aberrant expression has been reported in a number of pathological conditions where hypoxia is a major component, including tumors, myocardial infarction and cutaneous ischemic wound healing (*130*). Similar to breast cancer cells, it was shown that exosomes released from leukemia K562 cells grown in hypoxic conditions mediated the transfer of miR-210 in HUVECs, where it regulated expression of the target gene *EFNA3* and enhanced tube formation (*131*). Using an *in vitro* chronic hypoxia model of multiple myeloma, the same group demonstrated that exosomes derived from hypoxic cells increased tube formation of HUVECs (*132*). In addition to miR-210, these exosomes were enriched with miR-135b, which was transferred into HUVECs via exosomes to mediate their pro-angiogenic potential *in vitro* and *ex vivo* (plug assay). In HUVECs, exosome-transferred miR-135b regulated expression of the factor-inhibiting HIF-1 (*FIH-1*) target gene, resulting in increased HIF-1 transcriptional activity.

MicroRNAs, EVs and Radiation-Induced Nontargeted Effects

It is now widely accepted that radiation causes damage not only to directly exposed cells, but also to unirradiated cells in contact with, or at a distance from directly irradiated cells. These nontargeted effects, which include low-dose hypersensitivity, genomic instability, adaptive response, radiation-induced bystander effect (RIBE) and distant (abscopal) effect, are the consequences of intercellular communication between irradiated and unirradiated cells. RIBE refers to the biological alterations that occur in bystander cells and tissues, including DNA damage, gene expression alteration and apoptosis. Such communication is known to occur through cell gap junctions (*133*) or molecular signals released in the extracellular milieu by irradiated cells. Some factors responsible for mediating RIBE have been identified, including soluble cytokines such as transforming growth factor (TGF)-β, nitric oxide, and ROS (*134, 135*). Radiation-induced abscopal effects occur at a distance from the irradiated site and have been documented in preclinical and clinical studies as systemic effects of local radiotherapy for the treatment of different tumor types. However, while some studies have shown a systemic tumor-enhancing effect, others have shown that local irradiation inhibits distant tumor growth (*136*). *In vivo* studies using animal models investigated the molecular

TABLE 2
Extended.

Transferred miRNA	Recipient cells	Target gene inhibition	Biological effect	Reference
miR-223	HUVECs	*FBXW7*, *EFNA1*	n.d.	(*108*)
miR-142-3p	Immortalized mouse EC-like cells	Reporter activity	n.d.	(*109*)
miR-143, miR-145	Human aortic SMCs	*ELK*, *KLF4*, *PHACTR4*, *SSH2*, *MMP3*	Atheroprotective *in vivo*	(*110*)
miR-126	HUVECs	*RGS16*	Atheroprotective *in vivo*	(*114*)
miR-126	HCAECs *in vitro* and murine ECs *in vivo*	*SPRED1*	EC migration and proliferation *in vitro*; re-endothelialization *in vivo*	(*115*)
miR-126	HAECs	n.d.	Pro-angiogenic *in vitro*	(*119*)
miR-126	HUVECs	n.d.	Pro-angiogenic *in vitro* and *in vivo*	(*120*)
miR-150	HMEC-1	*MYB*	Increased cell migration *in vitro*	(*123*)
miR-214	HMEC-1	*ATM*	Reduced EC senescence *in vitro*; pro-angiogenic *in vitro* and *ex vivo*	(*124*)
miR-132	HUVECs	*p120RASGAP/RASA1*	Pro-angiogenic *in vitro*	(*125*)
miR-105	HMVECs	*TJP1/ZO-1*	Increased cell migration and vascular permeability *in vitro* and *in vivo*	(*126*)
miR-210	HUVECs	Reporter activity	Pro-angiogenic *in vitro*	(*127*)
miR-210	HUVECs	*EFNA3*	Pro-angiogenic *in vitro*	(*131*)
miR-210, miR-135b	HUVECs	*FIH-1*	Pro-angiogenic *in vitro* and *ex vivo*	(*132*)
miR-21	Unirradiated MRC-5 cells	*BCL-2*	RIBE	(*148, 149*)

mechanisms involved in the effect of radiation on distant, unirradiated tumor growth, and reported a role for p53 signaling and the immune system in mediating abscopal antitumor activity (*137, 138*). Since EVs can travel to distant sites because of their release in the blood circulation (*139*), the role of EVs and their cargo, in particular their miRNA content, as mediators of RIBE has been actively investigated recently. In fact, several studies have demonstrated RIBE on unirradiated cells in coculture with irradiated cells or upon exposure to CM harvested from irradiated cells, with involvement of miRNA expression variations. Using a transwell system, Chaudhry and Omaruddin showed that human lymphoblast TK6 cells underwent wide miRNA expression changes upon coculture with 2 Gy X-ray-irradiated cells (*140*). Using human non-small cell lung cancer (NSCLC) cell line H1299, the Yang team demonstrated increased ROS generation and p53 binding protein (53BP)-1 foci in cells exposed to CM harvested from 5 Gy X-ray-irradiated H1299 cells (*141*). These effects were TGF-β1 dependent and involved increased miR-21 expression in bystander cells. Of note, inhibition of miR-21 in bystander cells abrogated the biological effect induced by irradiated cells CM. Using a coculture system, the same group showed that human HaCaT keratinocytes irradiated with 0.56 Gy α particles induced micronuclei formation, elevated ROS levels and increased 53BP1 foci in unirradiated human embryonic dermal WS1 fibroblasts (*142, 143*). These effects were

mediated by activation of the TGF-β1-SMAD2 pathway in irradiated HaCaT cells, and led to increased miR-21 level in bystander WS1 cells, with possible involvement of SOD2. Interestingly, HaCaT cells that received up to 10 Gy X-ray irradiation failed to elicit RIBE in WS1 cells in this system, nor did they show activation of SMAD2, suggesting that RIBE is likely dependent on radiation quality, as well as the cell type under study. In another study, TGF-β1 in CM from 4 Gy X-ray-irradiated HeLa cells was also shown to account for the RIBE observed on recipient unirradiated HeLa cells, with increased 53BP1 foci and increased frequency of micronuclei (*144*). This effect was attenuated by induction of miR-663 in bystander cells, where it directly inhibited TGF-β1 in a feedback regulation mode.

It was recently shown that EVs (exosomes) could mediate short- and long-term RIBE in human MCF7 breast epithelial cancer cells exposed to CM from 2 Gy X-ray-irradiated cells, involving some RNA component as well (*145, 146*). Along this line, Jella *et al.* showed that exosomes released from HaCaT cells gamma-irradiated with 0.05–0.5 Gy (^{60}Co) induced increased cell death and ROS production in unirradiated cells (*147*). The Wang group demonstrated that CM from 2 Gy X-ray-irradiated human fetal lung MRC-5 fibroblasts mediated RIBE in unirradiated cells, with increased frequency of micronuclei and increased number of 53BP1 foci, as well as reduced clonogenic survival (*148*). Irradiated CM was enriched in miR-21, which was transferred to recipient bystander cells where it repressed

its target gene *BCL2* at the RNA and protein levels. Interestingly, transfection of miR-21 mimics into MRC-5 cells induced similar effects, suggesting a role for miR-21 in mediating RIBE. In a follow-up study, the same group demonstrated that exosomes isolated from irradiated CM could induce bystander effects by mediating miR-21 transfer from irradiated to bystander cells (*149*). Inhibition of miR-21 before irradiation blunted these effects, suggesting that exosome-mediated miR-21 transfer plays an important role in RIBE. Overall, these studies highlight the prevalent role of the TGF-β1 pathway and miR-21 for induction of RIBE.

Interestingly, miR-21 has been involved in vascular remodeling, where it was shown to be upregulated in SMCs after vascular injury. Notably, miR-21 regulated rat aortic SMC proliferation and apoptosis *in vitro* and in injured rat carotid arteries (*150*). miR-21 is also highly expressed in vascular ECs and is upregulated in a hypoxic environment (tumor, ischemic limb), leading to expression of *HIF1* and its target gene *VEGF*, thereby promoting angiogenesis. Furthermore, miR-21 and HIF-1 are involved in a hypoxia-induced positive feedback loop, in which HIF-1α drives miR-21 expression and miR-21 indirectly stabilizes and upregulates HIF-1α via inhibition of target gene *PTEN* (*148, 151*). Nonetheless, miR-21 has also been reported to inhibit angiogenesis by targeting *RHOB* in ECs and *HMGA2* in bone marrow-derived EPCs, leading to decreased cell migration and tube formation *in vitro*, and reduced angiogenesis *in vivo* in murine models of choroidal neovascularization and hind limb ischemia, respectively (*152, 153*). The effect of increased exosomal miR-21 released by irradiated cells on the vascular compartment remains to be explored, however.

Exosomes in Regenerative Medicine

Adult MSCs are multipotent stem cells that are routinely isolated from bone marrow or adipose tissue and expanded *ex vivo*. They have been extensively studied in the context of regenerative medicine, and they are currently evaluated in hundreds of clinical trials for a wide range of diseases, including cardiovascular diseases, graft-versus-host disease, cartilage and bone repair (*154*). Although the therapeutic capacity of MSCs was originally attributed to their differentiation potential into different reparative or supportive cell types, numerous studies have shown that the therapeutic benefit of MSCs essentially relied on their paracrine activity (*14*). Indeed, injection of MSC-CM could recapitulate MSCs beneficial effects in animal models of myocardium ischemia/reperfusion (*155, 156*) and cutaneous wound healing (*157, 158*). The Lim laboratory demonstrated that exosomes were the main contributor to the beneficial effect of MSCs paracrine activity for the treatment of myocardial injury (*159*). Since then, several studies have demonstrated the therapeutic efficacy of exosomes derived from different cell types (MSCs, ECs, adipose-derived

progenitor cells) in multiple pathological animal models, including kidney (*160*), heart (*159*) and lung (*161*) ischemia.

In addition, conditioned media (*162*) or EVs (*163*) from human cord blood-derived EPCs were shown to promote angiogenesis *in vitro* and *in vivo*. Similarly, the Losordo group demonstrated that exosomes derived from mobilized human CD34+ cells recapitulated the angiogenic activity of CD34+ cells, both *in vitro* and *in vivo* using Matrigel plug and corneal assays (*120*). Recently, a comprehensive proteomic analysis revealed that MSCs exposed to hypoxic conditions released exosomes with a robust profile of key angiogenic proteins, including PDGF, FGF, EGF and NFκB signaling pathway (*164*). Hypoxia preconditioned MSC-derived exosomes induced tube formation in HUVECs, which was completely abrogated upon treatment with an NFκB inhibitor. Our group is currently investigating the beneficial effect of MSC-derived exosomes for the treatment of radiological cutaneous injury. Preliminary results revealed a stimulation of angiogenesis and an improvement of skin quality after local exosome administration, raising new hopes in regenerative medicine for the treatment of radiation injuries (unpublished data).

A number of clinical trials aiming at using exosomes as therapeutic tools or vehicle for drug delivery have been published (*165*). For example, exosomes purified from dendritic cells pulsed with antigenic peptides were used as anticancer vaccines for two phase I clinical trials against melanoma and NSCLC (*166, 167*), and more recently for a phase II clinical trial against NSCLC (*168*). Furthermore, the immunosuppressive potential of exosomes was demonstrated by Kordelas *et al.*, who reported on the case of a patient with graft-vs.-host disease after allogeneic stem cell transplant for the treatment of acute lymphoblastic leukemia (*169*). The patient received allogeneic MSC-derived exosomes, resulting in attenuation of the symptoms and disease stabilization.

Altogether, these preclinical and clinical studies highlight the beneficial potential for exosome-based therapy with regard to angiogenesis and immunosuppression, and pave the way to the development of clinically compliant preparations of stem cell-derived exosomes for the treatment of radiological injuries.

CONCLUSION

An increasing number of studies highlight the contribution of EVs in signal transmission in a cell-to-cell communication process in different pathologies, including cardiovascular diseases and cancer. However, the extent of their contributions to patho/physiological processes and the mechanisms involved remain uncertain. An understanding of the molecular mechanisms governing EV formation, release and clearance, as well as those involved in cell-to-cell communication, will enable to us to envision new therapeutic strategies for limiting disease progression or

favoring tissue repair. Over the past decade, most efforts have been focused on elucidating the involvement of exosomes in radiation-induced nontargeted effects and the role played by miRNAs in these processes. Thus far, there is a lack of data on MPs in the field of radiation, which may be the result of current technical concerns and limited standardization. Nonetheless, a complete vision of the role of EVs, integrating MPs and exosomes in regulating vascular homeostasis in the irradiation field is necessary. In addition, one should also be aware that exosomes reflect only a partial picture of the vascular status and should not exclude the simultaneous contribution of the MPs derived from the cells of interest (Fig. 1).

An overview of the literature clearly indicates that research on EVs is still in its infancy and suffers from limitations. Despite the recent advances, the terms ''exosomes'' and ''extracellular vesicles'' or ''microvesicles'' have been used interchangeably in many published studies due to the current limited understanding of extracellular vesicle biogenesis, inconsistencies in extracellular vesicle purification protocols and a lack of thorough vesicle characterization. There remains an urgent need for clarification on a number of technical points, such as the EV nomenclature and the standardized protocols for their preparation. However, the complexity of EV heterogeneity and functions has to be taken into account. One of the most urgent challenges is to establish methods to separately characterize each kind of EV to precisely define their individual cargoes and functions, including their miRNA content. The first step in addressing this challenge is determining how to define and measure EVs in a reproducible manner. The characterization of MPs and other types of vesicles remains difficult because of their small size and heterogeneity in terms of phospholipid content and antigenic composition. In addition, there is no consensus on the best markers for defining ''rare'' EV subpopulations such as endothelium-, leukocyte-, and cancer-derived MPs, which appear to be the most relevant in terms of predictive value. While important work to standardize the pre-analytical and analytical variables has been achieved on behalf of the International Society on Thrombosis and Haemostasis Standardization Subcommittee in vascular biology for the MPs (170, 171), efforts in this area continue. Similarly, the International Society for Extracellular Vesicles provides researchers with a minimal set of biochemical, biophysical and functional standards that should be used to attribute any specific biological cargo or functions to EVs (12, 172, 173).

Another major field of optimization and standardization resides in the thorough analysis of EV RNA content. It is clear that the different EV subpopulations, exosomes, MPs, ABs, display distinct total RNA and miRNA profiles (118, 173). In addition, discrepancies within a subgroup, i.e., exosomal RNA profiles, have been observed among studies, which could be partly explained by differences in the cellular source as well as methodological parameters (175).

Several comparative studies highlighted the strong impact of the choice of both EV isolation method (176, 177) and RNA purification protocol (175, 178, 179), on downstream RNA profiling. In particular, comparison of RNA isolation protocols using phenol-based methods, column-based techniques or combined phenol/column-based approaches, revealed substantial variations regarding small RNA recovery and miRNA expression analysis in exosomes from cell culture and biofluids (175, 178, 179). To our knowledge, no consensus has been established so far, and the main recommendation remains to carefully choose the RNA isolation method according to the nature and amount of starting material, as well as the downstream experiments.

In this review we present information on the diverse implications of EV signaling in health and disease, how EVs may be utilized as diagnostic disease biomarkers and therapeutics and how their inherent properties potentially provide benefits over existing clinical interventions. Although the complexity of EVs naturally broadens their functional impact, this also makes investigations of their activity difficult. While the overall effect on a cell after EV uptake results from the combination of the particular EV-transferred components, including relevant angiomiRs, it is also determined by the cell of origin and its status at the time of secretion. In addition, it is also important to remember that different EVs can have synergistic or opposing effects on the recipient cell. Mechanistically, EVs participate in clearance of substances, information exchange and epigenetic modulation, and may even be responsible for the spreading of pathological proteins. This complex, multifunctional activity is explained by the highly heterogeneous content of EVs. All these regulatory functions have been delineated mostly using *in vitro* systems, although their *in vivo* relevance remains to be fully demonstrated. In the fields of radiation biology and oncology, a better understanding of EV biology together with standardized methods for EV quantification, isolation, storage and molecular characterization will greatly enhance the future promise for EV-based diagnostic and therapeutic applications.

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