Editorial

Angiogenic activity of exosomes isolated from human pericardial fluid

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Exosomes are endogenous, nanosized extracellular vesicles secreted by cells. Unlike microvesicles, which are released by direct budding from the surface membrane, exosomes originate in the endosomal compartment of a parent cell (1). As a result of this biogenesis, the exosome content is controlled by the cell packaging machinery, so that a portion of what a cell processes is integrated in the exosome payload (2). Exosomes carry bioactive molecules including proteins and RNA, circulate freely in all body fluids, and can deliver their molecular cargoes to recipient cells. We have, until recently, failed to wholly appreciate the function of EVs, and exosomes in particular, as mediators of intercellular communication.

The term exosomes was introduced by Johnstone and others in 1987 to designate particles released from cultured maturing sheep reticulocytes (3). These particles exhibited transferrin activity that was lost during reticulocyte-to-erythrocyte maturation. At that time, exosomes were mainly viewed as "garbage bins" by which a maturing cell eliminates factors from the plasma membrane that are no longer needed by a mature cell. Recent advances in our understanding of the paracrine mechanisms involved in many diseases including cancer have allowed us to realize the fundamental role of exosomes in intercellular cross talk. All examined eukaryotic cell types secrete exosomes in culture (4). *In vivo*, exosomes can be isolated from all body fluids, including blood, urine, cerebrospinal fluid, saliva, milk, ascites, bronchoalveolar lavage fluid, and pericardial fluid (PF) (5-12). The molecular content of vesicles isolated from these fluids reflects the activation and functional characteristics of their cells of origins, and therefore normal or pathological conditions. Hence, secreted vesicles can be used as markers of disease, as demonstrated prominently in cancer (13). Owing to their molecular content including proteins, mRNA and non-coding RNA [microRNA (miRNA) in particular], exosomes can elicit autocrine, paracrine, and endocrine effects on recipient cells and tissues. Exosomal miRNAs in particular can deeply regulate the transcriptome and function of recipient cells (14). Of note, miRNAs are viewed as attractive candidate biomarkers due to their relative stability and easy quantification from clinical samples.

PF contained in the pericardial sac surrounding the heart is one body fluid of potential interest to analyses of miRNA markers of heart disease. This fluid is formed by the active secretion of the pericardial cells and, in part, as a heart tissue filtrate (15). It has been shown that the concentrations of heart-specific hormones, such as atrial and brain natriuretic peptides and endothelin-1 are higher in PF compared to plasma, and that pathological conditions influence the cardiac hormone and growth factor composition of the fluid (16,17). Moreover, cardiac enzymes and troponins are present at higher levels in the PF than plasma, and increased levels of these molecules are useful in the post-mortem diagnosis of fatal myocardial infarction (18). miRNA profiling of PF samples from patients with heart failure identified, on the average, 256 miRNAs per sample. Seventy miRNAs out of 742 profiled miRNAs were detectable in all samples from 51 patients (19). The five most abundant miRNAs in PF were miR-21-5p, miR-451a, miR-125b-5p, let-7b-5p, and miR-16-5p. No specific signatures for cardiovascular pathologies or clinically assessed heart failure stages could be detected from the profiles and, overall, miRNA profiles of the samples were found to be very similar despite the heterogeneity in

the study population. It should be noted, however, that this study did not discriminate between miRNAs in the exosomal and non-exosomal fractions of PF. One other study (20) reported that miR-423-5p was enriched in PF compared to serum in patients undergoing cardiac surgery, and that its expression pattern was different from that of muscle- or vascular-enriched miRNAs (miR-133a, miR-126, miR-92a). This study suggested that serum miR-423-5p might be associated with unstable angina pectoris.

In the recent paper published in *Molecular Therapy*, Costanza Emanueli and her group (12) addressed the presence of exosomes in PF and their miRNA content, in comparison to miRNA expression in the myocardium and vasculature, in patients undergoing aortic valve surgery. The angiogenic activity of these vesicles was addressed. These investigators found that in comparison with peripheral plasma, PF contains exosomes enriched with miRNAs co-expressed in the myocardium and vasculature. Sixteen miRNAs of putative cardiovascular origin identified in PF were indeed well expressed in the myocardium and/or vasculature of the patients. Four (miR-21-5p, miR-451a, let-7b-5p, miR-16-5p) out of the 5 most abundant miRNAs previously identified in PF of heart failure patients (19) were among the 16 miRNAs of putative cardiovascular origin identified in PF-derived exosomes in this study in patients with aortic valve disease (12). A comparison of matched PF and plasma samples provided evidence supporting the hypothesis that PF is enriched with miRNAs released from cardiovascular tissues. These miRNAs were similarly detectable in the exosomal fraction of PF. For the majority of the individual miRNAs, differences in their concentrations in whole PF and whole plasma were reflected by similar differences between exosomes isolated from the two fluids. These findings supported the hypothesis that miRNAs produced by cells of the heart and central vessels are transported to PF, at least in part, via exosomes. The relative distribution of individual miRNAs between the exosomal and non-exosomal fractions of PF was highly variable suggesting a non-random incorporation of the miRNAs in the exosomes delivered to this fluid. The majority of these miRNAs were conjugated to the RNA-induced silencing complex (RISC) component Argonaute-2 (AGO-2). Another RISC component, miRNA processing enzyme Dicer, was also present within the exosomes isolated from the PF or peripheral plasma. When added to cultured endothelial cells kept under hypoxia, PF-derived exosomes inhibited apoptosis, increased cell proliferation, and promoted the formation of capillary-like endothelial cell networks on Matrigel. By contrast, plasma exosomes lacked such effects. The investigators then focused on the functional role of let-7b-5p, a member of the Let-7 family that includes other miRNAs known to stimulate angiogenesis (21), which was more abundant in PF than plasma and was present in PF exosomes. By transfecting endothelial cells with a let-7b-5p mimic or a let-7b-5p inhibitor, it could be demonstrated that let-7b-5p promotes angiogenesis in vitro. Moreover, let-7b-5p reduced the mRNA expression of one of three previously validated direct target genes, the anti-angiogenic TGFβ1 (22), leaving unchanged the other two potential target genes, LOX-1 and Caspase 3, in endothelial cells. To address the contribution of let-7b-5p on angiogenesis induced by PF-derived exosomes, Dicer knockdown (KD) was used to reduce the endogenous expression of miRNAs in vitro. Treatment with PF-derived exosomes restored intracellular let-7b-5p expression, which had been compromised by Dicer KD, consistent with transfer of let-7b-5p into endothelial cells via PF-derived exosomes. These vesicles restored the angiogenic capacity of Dicer KD-endothelial cells. let-7b-5p KD-exosomes transfected with the miRNA inhibitor could not restore let-7b-5p levels, inhibit TGFBR1 expression, or improve angiogenesis in recipient Dicer KD-endothelial cells. *In vivo*, the angiogenic potential of PF-derived exosomes was assessed in a mouse model of limb ischaemia. PF exosomes, but not plasma exosomes, improved post-ischaemic blood flow recovery, reduced the incidence of ischaemia-induced toe necrosis, and improved capillary density in ischaemic muscles. The delivery of PF exosomes into ischaemic limb muscles was associated with a trend toward increased let-7b-5p expression (P=0.18) and a significant decrease in TGFβ1 expression. These findings suggest that human PF-derived exosomes may promote angiogenesis both *in vitro* and *in vivo*, at least in part, via let-7b-5p transfer.

This study provides the first evidence that the pericardial space may represent a compartment in which miRNAs released from the heart are enriched compared with the peripheral circulation. Although plausible, the cardiac origin of individual miRNAs enriched in PF was not demonstrated directly, however. Most miRNAs are not cell type-specific but are expressed by many cell types, and differences in their expression levels between different cell types are relative. While there might be a theoretical rationale for using PF as a "liquid biopsy" sample, the usefulness of PF exosomes as biomarkers of heart disease remains to be investigated. Toward this goal, prospective studies collecting

clinical samples and data (at baseline and follow-up) will be required, as pointed out by the investigators. However, such studies face significant practical hurdles, as PF is not easily accessible except for patients undergoing heart surgery. Many heart diseases including ischaemic heart disease are associated with pericardial effusion only in a minority of the cases. In addition, non-surgical sampling of PF is not without risk. Thus, there are major practical limitations to the clinical translation of this approach. One possible exception may be the analysis of miRNAs in PF-derived exosomes in cancer patients with pericardial effusion, in whom PF sampling is routinely performed for diagnostic purposes. Within this clinical context, miRNA analyses of PF exosomes, in addition to cytological examination and chemical analyses, might theoretically assist in the diagnosis of malignant pericardial effusion, although this remains to be demonstrated.

Finally, this paper published by Emanueli's group (12) identified let-7b-5p as a proangiogenic miRNA while also demonstrating proangiogenic activities of PF-derived exosomes in an *in vivo* model of limb ischaemia. Of note, exosomes released from other cell types including cardiac progenitor cells (23), mesenchymal stem cells (24), and endothelial cells (25) likewise exhibit proangiogenic activity. However, exosomes with anti-angiogenic properties have also been described. For example, exosomes secreted by cardiomyocytes from adult Goto-Kakizaki rats, a commonly used animal model of type 2 diabetes, inhibited proliferation, migration, and tube-formation by mouse cardiac endothelial cells in a miR-320-dependent manner, whereas exosomes secreted by cardiomyocytes from control Wistar rats induced the opposite effects (26). Thus, not only the parent cell type but also its functional status and systemic disease conditions may influence the biological activities of the secreted vesicles. In this regard, the investigators appropriately pointed out that, in addition to let-7b-5p and other proangiogenic miRNAs, PF exosomes also contained several anti-angiogenic miRNAs, and that the overall proangiogenic responses to the PF exosomes might be dictated by a functional prevalence of proangiogenic miRNAs that are simultaneously transferred by these vesicles. How these responses change under pathological conditions (e.g., whether or not they are accentuated in the presence of myocardial ischaemia as a proangiogenic stimulus) remains to be investigated.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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