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Extracellular Vesicles in Cancer: Purpose and Promise

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Abstract

Extracellular vesicles refer collectively to a heterogeneous group of membrane-bound vesicles released from cells and loaded with bioactive proteins, nucleic acids, and lipids. The concept of extracellular vesicles has rapidly evolved from once being viewed as cellular debris to their recognition as packets of cellular information with considerable promise for clinical applications as biomarker platforms and therapeutic vehicles. These shed vesicles have emerged as critical mediators of intercellular communication in both local and distant microenvironments during normal physiological processes, as well as in orchestrating systemic pathophysiological events in disease. This mode of cellular crosstalk is particularly relevant to modulating the tumor microenvironment and orchestrating paths of least resistance during metastases. Here we describe recent advances for the roles of extracellular vesicles in tumor progression and their potential as disease biomarkers.

Introduction

Emerging evidence reveals an extended spectrum in the modes of intercellular communication that include not only soluble factors secreted by cells but also actively shed membrane-bound vesicles containing bioactive proteins, lipids, and nucleic acids. These latter particulate sacs, collectively referred to as extracellular vesicles (EVs), are universally produced by all cells, vary in cargo content and have unique modes of biogenesis. The amount released as well as the composition can vary in response to environmental stimuli^{1,2}. EVs include exosomes, which are particles ranging from 50 to 80 nm in diameter and formed from multi-luminal multivesicular bodies; microvesicles, characterized as more heterogeneous vesicles that range in size from 200 nm to several microns; and apoptotic bodies which are generated from dying cells and are typically larger than a micron in diameter^{3,4}. Oncosomes are large EVs produced from tumor cells whose cargo and function appear to overlap with microvesicles⁵. More recently 12 nm nanovesicles have also been described⁶. While there has been some controversy in the nomenclature of different types of vesicles, the basic criteria of EVs have been established. The development of methods to separate these different subtypes of EVs is necessary and will greatly enhance further research in this field.

EVs are thought to function as information carriers that modulate the extracellular environments into which they are released. This includes EV cargo modulating the

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phenotype and behavior of recipient cells they interact with. These interactions include ligand receptor binding, signaling cascades in target cells, EV-plasma membrane fusion and uptake of EVs into recipient cells by endocytosis^{3,7}. Studies have shown that the fate of the endocytosed EV can entail the release of EV cargo into the cytoplasm and/or transport to the nucleus or other organelles, all of which can bring about functional changes in recipient cells.

EVs have been implicated in numerous pathological conditions and most especially in cancers^{8–10}. This review will highlight recent developments, focusing on the roles of EVs in tumor progression and the utility of EVs as cancer biomarkers.

EVs in Tumor Progression

Tumor-derived extracellular vesicles (TEV) carry a versatile array of bioactive cargo ranging from nucleic acids (DNA, miRNA, mRNA etc.) to metalloproteases, metabolites, and lipids^{1,9}. It is the wide spectrum of TEV cargo that enables their multitude of functional roles during cancer pathogenesis⁹. TEVs function in three primary roles during disease development by influencing the tumor microenvironment, exerting effects within the circulation, or through their ability to traverse long distances to alter distal sites. In each of these roles, TEVs have emerged as exceptionally capable of establishing and maintaining a pro-tumorigenic condition.

EV-mediated communication: it's all about the cargo

During primary tumor growth, tumor cells, acting in part through secreted EVs, are subject to reciprocal communication between the plethora of components that make up the multicellular tumor ecosystem. Tumor cells release large quantities of extracellular vesicles enriched with functional protease cargo utilized to remodel the extracellular matrix (ECM) and facilitate tumor cell invasion^{8,11–13}. Membrane type I matrix metalloprotease (MT1-MMP, or MMP14) for example, is the protease component primarily responsible for the invasive capacity of the tumor microvesicle population and its inclusion is required for robust microvesicle-mediated invasion⁸. Similarly, exosomes release has been reported to facilitate degradation at site at sites of invadopodia-mediated invasion¹². Tumor cells are able to switch between the use of microvesicles and invadopodia to facilitate invasion through extracellular matrix, and that this behavior is governed by antagonistic signaling through RhoA and Rac1, as well as the physical properties of the extracellular matrix¹⁴. Enhanced invasion stemming from TEV-mediated ECM remodeling extends beyond the direct proteolytic degradation of matrix components, as TEV cargo components are capable of activating additional intrinsic proteolytic machinery in the extracellular milieu. Exosome-mediated secretion of Hsp90 α , for example, increases tumor cell motility and invasion through the tissue plasminogen activator mediated activation of the extracellular protease plasmin¹⁵. In addition to acting on the ECM, secreted TEVs have been demonstrated to transfer cargo contents and phenotypic identity to recipient cells.

Lipid bilayer enclosed TEVs serve as potent means to facilitate this non-canonical form of cell-cell communication in which tumor cells can modulate surrounding stromal cells through the horizontal transfer of bioactive, and sometimes pathogenic molecular cargo¹⁶.

The functional transfer of pathogenic TEV components has been observed *in vitro*. In a series of elegant experiments, Al-Nedawi and colleagues demonstrated the propensity for glioblastoma-derived vesicles to transfer the oncogenic variant of the EGF receptor (EGFRVIII) to propagate the malignant potential of the shedding tumor cells¹⁷. More recently, using a novel organoid culture system, the addition of esophageal adenocarcinoma derived extracellular vesicles was found to induce a neoplastic phenotypic response¹⁸. In this study, TEV uptake resulted in increased proliferation; wall thickening and lumen shrinkage; loss of endogenous PTEN; and increased cell survival relative to control cells. These changes resulted at least in part from the TEV-mediated transfer of miR-25, and miR-210.

The most well-characterized EV-mediated nucleic acid transfer is exosomal shuttle RNA (esRNA) which it is now known, represents a differential pool of RNA relative to the secreting cell, highlighting the likelihood that specific small RNA is loaded into released TEVs^{16,19–22}. The selective enrichment of TEV RNA cargo is indicative of the pro-tumorigenic nature of TEVs. Numerous reports have outlined contributory roles for TEV RNA in drug resistance, angiogenesis, modulating immune surveillance, and inducing cell proliferation⁹. In addition to TEV mRNAs which are subsequently translated into functional proteins, TEV miRNA, for example, may be transferred to recipient cells, where it is capable of post-transcriptionally regulating gene expression and reprogramming recipient cells to facilitate tumorigenesis^{23,24}. It is tempting then, with the identification of putative oncomiRs as TEV cargo, to speculate on the oncogenic potential of TEVs themselves, or as yet unidentified TEV subpopulations²³.

Promoting metastases: EV's induce vascular effects

Metastasis, though responsible for upwards of 90% of all cancer-related deaths, is not merely a cancer-cell phenomenon. It is a complex cascade of events, which stem from the collective effects of alterations to the tumor and the surrounding environment. In order to metastasize, tumor cells must be able to penetrate the endothelial cell barrier and enter the circulation. Once in the circulation, TEVs exert a pro-thrombotic, pro-tumoral response which correlates with poor prognosis for tumor patients^{25,26}. Furthermore, the clotting initiating tissue factor (TF) is widely expressed on multiple tumor types, and its levels reported to increase with advancing disease stage^{27,28}. *In vitro* incubation of washed platelets with TEVs released by the highly invasive MDA-MB-231 cell line resulted in increased platelet p-selectin exposure and platelet aggregation when compared to less invasive MCF-7 derived TEVs. MDA-MB-231 TEVs contained higher levels of TF protein, increased TF-dependent pro-coagulant activity, and TF-dependent platelet aggregating ability than the less invasive counterparts²⁹. *In vivo* ovarian clear cell carcinoma tumors, a sub-type with a particularly poor prognosis, express high levels of TF, often in complex with coagulation factor VII. Interestingly, clear cell carcinoma cells have been found to release higher levels of TF containing TEVs than the previously mentioned MDA-MB-231 cell line, and in xenograft mice bearing these tumors, renders the blood hypercoagulable³⁰.

Beyond promoting a pro-tumorigenic, hypercoagulable condition within the blood, circulating TEVs function to fundamentally alter the vasculature itself. Hypoxia, which itself is a common feature of tumors and plays a central role in disease progression, potentially

induces TEV release as a means to stimulate angiogenesis^{31,32}. Among the changes induced under hypoxic conditions is an increase in the TME mediated release of the pro-angiogenic miR-210 in part through hypoxia-induced upregulation of neutral sphingomyelinase²³³. Recently, it was reported that TMEs isolated from bulk prostate cancer cells displayed differential microRNA content relative to cancer stem cells generated from the same primary cell cultures. Among those differentially expressed, in particular those more abundant in the cancer stem cell population, were miR-20-5p, miR-17-5p, and miR-21-5p which function to positively regulate angiogenesis³⁴. In addition to stimulating the development of neovascularization required to feed growing tumors, TMEs, through their interactions with endothelial cells, are capable of altering the existing vasculature. Metastatic breast cancer cells release TMEs containing abundant miR-105 which targets the important tight junction protein Zonula Occludens 1 (ZO-1) in recipient cells³⁵. Loss of ZO-1 leads to increased vascular permeabilization and primes the site for invasion and metastatic outgrowth.

EVs contribute to establishing pre-metastatic niches

A modern derivation of Paget's "seed and soil" hypothesis, the theory of the pre-metastatic niche suggests that tumors are able to prime the secondary site before metastatic cells can effectively colonize it, and TEVs have emerged as powerful weapons due their myriad bioactive cargoes. Visual evidence of TME transfer between primary tumors and has been demonstrated by visualizing GFP-exosome migration between tumor and normal cells *in vitro* and between tumor and stromal cells in orthotopic nude mouse models providing strong evidence that TMEs serve as long-distance messengers³⁶. Additional reports have highlighted the role of TMEs from CD105 positive renal cancer cells in triggering angiogenic pathways in recipient lung endothelial cells through the transfer of RNA cargo regulating MMP2, MMP9, and VEGF³⁷. Similarly miR-494 and miR-542, when delivered via TMEs to lung or lymph node stromal cells led to elevated expression of a panel of proteases including MMP2 and MT1-MMP, along with the downregulation of the cell-cell junction protein cadherin-17³⁸. Upon arrival at distal sites, tumor cells are in constant competition with native cells for available nutrients. Tumor cells can utilize TEVs to alter the metabolic landscape to favor tumor outgrowth either through the direct release of TEV miRs or by stimulating the release of pro-tumorigenic TEVs from cancer associated fibroblasts. TEV-associated miR-122 targets pyruvate kinase in recipient cells, reducing glucose uptake in the stroma and increase nutrient availability to support metastatic outgrowth. Similarly, prostate cancer associated fibroblasts were shown to target the oxidative phosphorylation pathway by releasing TEVs containing miR-22, let-7a, and miR-125b allowing for increased prostate cancer glycolysis and driving glutamine into the TCA cycle.

A salient feature of the formation and conditioning of a pre-metastatic niche is the tumor-specific preferential colonization of certain organs during metastatic outgrowth. Hoshino and colleagues recently demonstrated the ability of tumor exosomes to determine organotropic metastasis by conditioning specific organs in an integrin dependent fashion³⁹. Following fusion with recipient cells, the tumor exosomes initiated pre-metastatic niche formation through Src activation and pro-inflammatory S100 gene expression. A similar mechanism is believed responsible for the organotropic affinity of lung cancer cells to the bone marrow

following the EV-mediated transfer of the surface integrin CD41, highlighting the importance of EVs and the intercellular transfer of molecular cargo during cancer pathogenesis⁴⁰.

EVs as platforms for cancer biomarkers

The release of EVs into body fluids such as blood and urine lends to their promise as circulating biomarkers in the surveillance of disease progression. EV cargo, both proteins and nucleic acids, is likely indicative of molecular changes in the tumor. For example, EVs have been detected in the serum of glioblastoma patients and depletion of circulating EVs correlates with the removal of the tumor⁴¹. EVs have also been detected in the circulation of patients with breast, lung, ovary, prostate, colorectal gastric and other cancers^{3,42}. EV nucleic acids have piqued high interest as tumor biomarkers. Since the original discovery of mutant EGFRvIII mRNA in tumors drew attention to its possible use as a biomarker for brain tumors like glioma⁴¹, the list of EV nucleic acids that can be used for these purposes have been growing and include changes to the EV transcriptome^{43,44}, small non coding RNAs in sera⁴⁵, and elevated levels of specific microRNAs^{46,47}. Studies showing that exosomal RNAs are protected from RNase-dependent degradation and thus can be stably detected in circulating plasma and serum^{48,49}, make them strong candidates for further testing as potential biomarkers. Further, miRNA (miR) patterns in circulating TEVs in lung adenocarcinomas and tumors are different from those detected in control subjects⁵⁰. EV-associated miR-141 and miR-375 correlate with tumor progression in prostate cancer^{48,51}. MiR-21 and miR-1246 levels in circulating TEVs are reported to be elevated in the serum of patients with esophageal squamous cancer compared to patients with benign tumors^{52,53}.

An increasing number of EV proteins have been targeted as potential candidates for cancer diagnostics⁵⁴. For example, CD63, a member of the tetraspannin family is present at higher levels in TEVs shed from malignant tumor cells relative to non-tumor cell types⁵⁵. EVs shed from brain tumors contain elevated levels of EGFRvIII and TGF- β ^{41,56}. Smalley et al identified several EV proteins including Gs protein and retinoic acid induced protein, from the urine of patients as potential biomarkers for bladder cancers⁵⁷. Subsequently, Chen et al identified 24 urinary EV proteins at levels altered in bladder cancer relative to a control patient cohort (with hernias)⁵⁸. These proteins hold great potential as tumor diagnostics. TEVs from human ovarian cancers have been shown to contain claudin, ADAM 10 and CD24 is elevated amounts^{59,60}. In another study, the levels of CA125, along with the proteins ARF6, VAMP3, and MT1-MMP were concentrated in a subset of larger EVs isolated from the ascites of ovarian cancer patients relative to their level in whole ascites⁸. High-resolution point of care technologies have been developed to detect and monitor levels of serum EVs with specific cargos⁶¹. Incorporation of deep sequencing technologies will only enhance the sensitivity of detection. Thus, TEVs hold significant promise as biomarker platforms.

Summary

Accruing research supports the importance of EVs in intercellular communication during tumor progression. TEVs serve to modulate the tumor microenvironment by their release

from donor cells and uptake by recipient cells. This mode of intercellular communication provides another dimension of cellular crosstalk wherein recipient cells are instructed to evoke specific cellular behaviors that may be transient or persistent. Mechanistically TEVs serve to promote cell invasion, information exchange and epigenetic modulation all of which can temper and modify the genotype and phenotype of target cells. Their inherent presence in various body fluids is being harnessed for potential diagnostic application. The potential use of EVs is still at an exploratory stage and their promise as multi-panel biomarkers, encompassing proteins and nucleic acids, possibly EV lipids⁶², remain to be fully explored.

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Figure 1.

Tumor-derived extracellular vesicles mediate the establishment and maintenance of a pro-tumorigenic condition. TEVs, through their myriad bioactive cargoes act to promote the growth and invasion of primary tumors. Upon infiltration of the vasculature, additional systemic effects are observed including alterations to the blood's innate ability to coagulate. The development of leaky vasculature contributes to the dissemination of TEVs into tissues where they prime sites for metastatic colonization and outgrowth.