Implications of Extracellular Vesicle Transfer on Cellular Heterogeneity in Cancer: What Are the Potential Clinical Ramifications?
Anoek Zomer and Jacco van Rheenen

Abstract

The functional and phenotypic heterogeneity of tumor cells represents one of the greatest challenges in the successful treatment of cancer patients, because it increases the risk that certain individual tumor cells possess the ability to, for example, metastasize or to tolerate cytotoxic drugs. This heterogeneity in cellular behavior is driven by genetic and epigenetic changes and environmental differences. Recent studies suggest that an additional layer of complexity of tumor heterogeneity exists, based on the ability of cells to share functional biomolecules through local and systemic transfer of extracellular vesicles (EV), with profound effects on cellular behavior. The transfer of functional biomolecules between various populations of tumor cells and between tumor cells and nontumor cells has large consequences for both the tumor cells and the microenvironment that support the cellular behavior of tumor cells, and therefore for the clinical outcome of cancer. Here, we discuss the latest findings on EV transfer and the potential implications of EV-mediated local and systemic transmission of phenotypic behavior, particularly in the context of tumor heterogeneity, metastatic disease, and treatment response. Cancer Res; 76(8); 2071–5. ©2016 AACR.

Introduction

Most cancers consist of a complex mixture of tumor cells, nontransformed stromal cells and noncellular factors including growth factors and the extracellular matrix (1). Tumor cells themselves also display a large genetic heterogeneity due to genomic instability that leads to an increased mutation rate and the emergence of different somatic aberrations (2). The behavior of individual tumor cells is not solely determined by genetic alterations. Heterogeneity between tumor cells can be further increased due to regional differences in the tumor microenvironment (TME), for example, in hypoxia or the presence of growth factors and the extracellular matrix (1). Tumor cells also display a large genetic heterogeneity due to genomic instability that leads to an increased mutation rate and the emergence of different somatic aberrations (2).

Soluble factors such as secreted ligands that can bind plasma membrane receptors of both tumor cells and stromal cells have been extensively studied and are considered to be the central players in the communication between different cell types. For example, it has been shown that (epi)genetically distinct tumor cell subsets enhance tumor growth (6, 7) or invasion (8) by transferring soluble proteins from one cell population to another. In recent years, it has become increasingly clear that cells also use other mechanisms to facilitate intercellular exchange of molecular information (12). An early finding that suggest that EVs influence tumor heterogeneity, thereby regulating multiple aspects of tumor progression, and we discuss the potential clinical implications.

Cell–Cell Communication through the Transfer of Extracellular Vesicles

Almost all cells release different types of extracellular vesicles. These are typically classified as either microvesicles, which bud from the cellular plasma membrane, or exosomes, which originate from multivesicular bodies and shed upon fusion of multivesicular bodies with the plasma membrane (12). Since it is not clear yet whether the various vesicles that are present in an in vivo microenvironment have distinct or overlapping functions, we use the generic term extracellular vesicles (EV) in this review. In cancer patients, tumor-derived EVs have been found in diverse body fluids such as blood (13) and ascites (14). It is increasingly evident that EVs have a key role in regulating cell–cell communication through the transfer of molecular information (12). An early landmark paper showed that EVs released by mast cells contain messenger (m)RNA molecules, and that these transcripts can be transported to recipient cells where they are translated into...
functional proteins (15). Besides mRNA, EVs are now known to shuttle many other functional biomolecules, including proteins, lipids, microRNA (miRNA), and DNA, between different cell types present in multicellular organisms (12). Transfer of vesicular content can influence the phenotypic behavior or fate of recipient cells, for example, by inducing differentiation or de-differentiation, or by promoting apoptosis (reviewed in ref.16). Although it is currently not well understood how long EV-mediated effects last, the duration may be dependent on the type of biomolecules being transferred. Possibly, vesicular transfer of DNA, miRNA, mRNA, and/or transcription factors leads to epigenetic reprogramming of recipient cells, resulting in a stable behavioral change. Conversely, EV-mediated transfer of, for example, membrane receptors, proteins that are considered to actively turnover, may only alter the phenotype of recipient cells for a short period of time.

**Studying the Uptake of EVs Released by Experimentally Defined Cells**

Accumulating in vitro evidence suggests that EVs contribute to the heterogeneous nature of tumors by transferring molecular information between different cell types or cells with differential behavior. However, it has been challenging to fully characterize these individual cells in an in vivo setting, because in vivo only a few cells may take up EVs and no techniques existed to distinguish those cells from the vast majority of cells that do not take up EVs. Moreover, since virtually all cells produce EVs, it has been challenging to study the consequence of the uptake of EVs that are produced by specific cell types. Recently, the Cre-LoxP system was utilized to overcome these technical limitations and to directly identify tumor cells that are exposed to the content of EVs released by a researcher-defined population of cells in living mice (17–19).

In this Cre-LoxP system, cells express a reporter construct that switches from DsRed to eGFP expression upon the functional transfer of Cre-recombinase (Cre) from Cre-expressing cells (19). Cells that have and have not taken up EVs from Cre-expressing cells can be discriminated on the basis of their color (green versus red) and therefore this system allows the identification of reporter cells that are exposed to the cargo of only the EVs released by Cre-expressing cells. Differential behavior of these two cell populations (that have or have not taken up EVs) can be studied to unravel functional implications of EV transfer. Importantly, and in contrast with other reporter systems that make use of fusing fluorescent proteins to EV and/or membrane markers (20, 21), the Cre-LoxP system reports both the uptake and the functional release of the cargo of EVs. Furthermore, the Cre-LoxP method provides the ability to detect several modes of Cre exchange between Cre-expressing cells and reporter-expressing cells including cell–cell contact, although to our knowledge it has, thus far, only been used to study EV exchange (17–19). We have recently used this method to show that more malignant cells phenocopy their behavior to less malignant cells through EV transfer in living mice (19).

**Phenocopying of Malignant Behavior through the Transfer of EVs**

High-resolution intravital microscopy has been extensively used to study the heterogeneous behavior of cancer cells within tumors (5). We used intravital microscopy to study the release of EVs from tumor cells in vivo and showed that highly migratory MDA-MB-231 breast tumor cells release a heterogeneous population of small and large EVs into the TME (19). Next, we isolated these EVs and tumor cells and identified that the mRNAs of around 200 genes involved in metastasis are enriched in these EVs. Because it has already been shown that heterogeneous tumors consist of subpopulations of cells with differing metastatic potential (22), we questioned whether the ‘malignant’ message encoded in EVs released by highly migratory tumor cells could be transmitted to other, less aggressive tumor cells. To address this, we used the Cre-LoxP system and tested whether the less malignant T47D reporter cells report the uptake of EVs released by Cre-expressing highly malignant MDA-MB-231 cells (19). Interestingly, we observed that EVs released by MDA-MB-231 cells are taken up by the T47D cells located within the same tumor and within distant tumors, suggesting local and systemic EV transfer. Next, we combined intravital imaging to visualize cellular behavior of individual cells and the Cre-LoxP system to distinguish between cells that did or did not take up EVs released by Cre-expressing cells. By intravital imaging, we showed that T47D cells that report the uptake of EVs released by the aggressive Cre-expressing MDA-MB-231 cells display an increased migratory behavior compared with T47D cells that do not report the uptake of these EVs. Since migration is one of the limiting steps in the metastatic cascade, we next tested whether metastasis was also affected. In line with the enhanced migration, we found that the capacity to form lung metastases increases for those T47D cells that report the uptake of EVs released by the highly metastatic cells compared with their counterparts that did not take up EVs. These findings suggest that within primary tumors, cells with high metastatic potential transfer EVs to less metastatic cells, thereby equipping them with migratory properties and allowing these cells to leave the primary tumor.

**EV Transfer May Contribute to Tumor Progression by Directing the Behavior of Multiple Cell Types**

The existence of in vivo EV transfer between tumor cells and between tumor and nontransformed cells (18, 19) adds another layer of complexity to the concept of cell–cell communication and tumor heterogeneity. The functional effects of EV transfer between different cell types present in tumors have been extensively investigated in vitro. First, EVs may mediate cross-talk between different subsets of cancer cells. A key study showed that a highly aggressive population of glioma cells, expressing a truncated and oncogenic form of mutated EGFR (EGFRVIII), can transfer this protein to EGFRVIII-negative glioma cells via EVs (23). This transfer leads in the recipient cells to an increased oncogenic activity including activation of the mitogenic MAPK and survival Akt pathway, and an increase in anchorage-independent growth. Similarly, Higginbotham and colleagues showed that tumor EVs containing EGFR ligands can increase the invasiveness of recipient cancer cells (24). Second, EVs released by tumor cells have been shown to be capable of transforming healthy nontumorigenic cells. For example, breast cancer EVs containing RISC-associated miRNAs mediate silencing of target mRNAs in nontumorigenic recipient cells, thereby changing the transcriptome of these cells and instigating malignant transformation (25). Other studies have shown that tumor EVs can be transferred to nontumorigenic stromal cells such as endothelial cells or immune cells to shape the TME and accelerate tumor progression. In vitro evidence suggests

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that EVs released by tumor cells have the potential to induce angiogenesis (26, 27) and inhibit antitumor immune responses (reviewed in ref.28). These results have been recently validated in \textit{in vivo} experiments using the Cre-LoxP system, showing that tumor EVs are taken up by myeloid-derived suppressor cells (MDSC) present in the local TME (18). MDSCs that take up these EVs display enhanced immunosuppressive properties compared with MDSCs that do not take up tumor EVs. Third, several studies demonstrated functional EV transfer from stromal cells to tumor cells. For example, EVs released by fibroblasts and activated T cells have been shown to increase the invasive phenotype of tumor cells (29, 30).

Collectively, these studies suggest that EV transfer between different cell types (transformed and nontransformed) can influence tumor progression. However, it is important to validate these findings under physiologic conditions where both EV release and EV uptake occur in an \textit{in vivo} setting. Recent reports, showing EV exchange between tumor cells and CD45+ leukocytes or CD45− cells in living mice by means of the Cre-LoxP system (18, 19), illustrate the great potential of this approach to further characterize the molecular and functional effects of \textit{in vivo} EV transfer between the many different cell types that are present in heterogeneous tumors and in distant organs (Fig. 1).

**Systemic EV Transfer**

EVs released \textit{in vivo} by primary tumor cells have been shown to travel through body fluids, in this way conveying functional information to physically separated primary tumor cells and to distant nontransformed stromal cells (Fig. 1). A few pivotal studies showed that systemically administrated tumor EVs can modulate distant stromal cells to facilitate metastatic outgrowth in distant organs (31–36). First, EVs released from highly metastatic melanoma cells were shown to prepare a premetastatic niche in the lungs and bone (31). Upon injection in naive mice, these EVs are taken up by bone marrow-derived cells, resulting in the “education” of these cells by increasing their amounts of MET and activated phospho-MET. The reprogrammed bone marrow-derived cells display a provasculogenic phenotype and mobilize to distant organs to prepare them for the arrival of tumor cells by promoting angiogenesis formation. A second study by Lyden and colleagues showed that pancreatic cancer-derived EVs can prime the liver for metastatic spread (32). EVs derived from a pancreatic cancer cell line are taken up by liver-resident Kupffer cells with subsequent effects on their gene expression profile. In response to an increased TGFβ release by Kupffer cells, hepatic stellate cells increase the production of fibronectin, enhancing the recruitment of macrophages from the bone marrow. These pancreatic cancer EVs-induced changes in the liver microenvironment facilitate the outgrowth of tumor cells that escape from the primary tumor and increased liver metastatic burden. These results are supported by a study showing that cancer-derived EVs can be taken up by distant macrophages that subsequently activate NF-κB and secrete proinflammatory cytokines (33). Another recent paper showed that tumor-derived EVs can suppress glucose uptake by non tumor cells in the premetastatic niche thereby increasing nutrient availability for metastasized tumor cells (34). In addition to these studies showing that tumor-derived EVs can create a premetastatic niche by instructing stromal cells to create a favorable environment for arriving tumor cells, other evidence indicates that EVs released by tumor cells can promote destruction of the vasculature in distant organs, including the blood–brain barrier, thereby facilitating the migration of tumor cells through these natural barriers (35, 36).
Taken together, these studies have revealed that tumor-derived EVs can modulate distant stromal cells to favor the outgrowth of metastases. Recent experiments utilizing the Cre-LoxP method confirmed these results by showing that nontransformed cells in the lungs, spleen and lymph nodes take up EVs directly released from tumor cells in vivo (19). Future studies should determine whether the potential of EVs to travel far from their site of origin is only relevant for creating a premetastatic niche or whether this feature also contributes to later stages of metastasis formation.

**Potential Clinical Implications of Systemic EV Transfer**

A vast amount of literature suggests that complex and bidirectional interactions occur between the primary tumor and metastatic foci (37). For example, a number of clinical case studies suggest that surgical removal of the primary tumor can accelerate metastatic development (e.g., refs. 38, 39). Although serum transplantation experiments between animals provided evidence for a serum growth factor being responsible for communication between primary tumor and metastatic cells (40), the exact molecular mechanism underlying the clinical observations is still unknown. Potentially, part of this cross-talk could occur through systemic transfer of EVs (Fig. 1). Tumor cells that are able to metastasize to a secondary site may transfer EVs and increase the metastatic capacity of other tumor cells in the primary tumor, thereby creating a feed-forward loop reinforcing metastasis formation. In line with this idea, two recent studies showed that systemic delivery of EVs derived from highly metastatic tumors can enhance metastasis formation of otherwise weakly metastatic cells (19, 41). Conversely, EVs released by less-malignant primary tumor cells may slow down metastatic outgrowth, thereby explaining clinical observations that report an acceleration of metastatic outgrowth upon removal of the primary tumor (37). Intravital imaging experiments demonstrated that cancer cells that have taken up EVs released by weakly metastatic tumor cells display a small decrease in in vivo migration compared with cells that did not take up these EVs, indicating that less malignant behavior also has the potential to be phenocopied through the transfer of EVs (19).

**Anticancer Therapies Are Potentially Influenced by EV Transfer**

A relatively new area of investigation involves the relationship between EV transfer and response to anticancer treatments, and so far in only a few studies, this clinically relevant subject was approached. EVs can mediate drug resistance in a direct manner through uptake of drugs in vesicles thereby limiting the bioavailability of the drug to tumor cells. Especially under low pH conditions, tumor cells release cisplatin-containing EVs, resulting in lower amounts of cisplatin in tumor cells (42). Apart from the sequestration of drugs in vesicles, EVs can be involved in mediating drug resistance in an indirect manner by transferring biomolecules to recipient cells that direct these cells toward a drug resistant phenotype. *In vitro* experiments showed that chemoresistant cells can confer resistance on chemosensitive cells through EVs by, for example, stimulating multidrug efflux transporter P-glycoprotein production (43, 44) or by stimulating the prosurvival AKT/mtTOR pathway (45). Stromal cells present in the TME have also shown to promote tumor cell survival after radiation or chemotherapy through the transfer of EVs (46).

It is not extensively explored whether functional biomolecules can be transferred through apoptotic bodies, a type of EVs formed upon programmed cell death, thereby mediating cross-talk between the many different cell types in tumors especially upon therapy. Apoptotic bodies were previously considered as “waste bags” containing the expelled content of dying cells, but findings from the last decade challenged this notion as it became clear that also apoptotic bodies are engulfed by other cells with functional effects on behavior. For example, two studies showed that apoptotic bodies released by transformed cells can horizontally transfer DNA to fibroblasts and endothelial cells and that the DNA can be propagated upon division of recipient cells, provided that the cells are p53 negative (47, 48). As in 50% of all human tumors, the p53 gene is mutated or deleted, DNA transfer through apoptotic bodies may be extremely relevant in a tumor setting, and may have profound consequences for tumor progression. Especially upon treatment, dying tumor cells may spread DNA and mutations to surviving tumor cells, thereby accelerating the process of tumor evolution. Also, it has been shown that concomitant transfer of tumor DNA with genes that inactivate the p53 pathway allows tumor DNA propagation in nontransformed cells (48), indicating that apoptotic tumor cells could affect the behavior of nontransformed cells. However, in-depth *in vivo* studies are essential to test such hypotheses in a physiologic setting and to fully understand the clinical implications of the transfer of apoptotic bodies.

**Concluding Remarks**

A plethora of studies that used *in vitro*-isolated EVs emphasize the importance of EV-mediated cross-talk between cells in tumor progression and response to anticancer treatments. These data in combination with the recent *in vivo* evidence of EV transfer (18, 19, 31, 32) revolutionized our ideas on cell–cell communication in heterogeneous tumors. Future studies, for example, using the Cre-LoxP system, should focus on validating *in vivo* findings under physiologic conditions where both EV release and EV uptake take place in an *in vivo* setting. Moreover, future studies that will reveal the molecular mechanisms of EV loading, release and uptake of the various subtypes such as microvesicles and exosomes, will further help to investigate the role and relevance of EV transfer in cancers. Together, these findings will significantly contribute to understanding the complex communication mechanisms that take place in heterogeneous tumors and will determine the clinical implications of EV transfer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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