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Molecular Pathways: Tumor-derived microvesicles and their interactions with immune cells *in vivo*

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Abstract

Cancer is not merely a cell-intrinsic genetic disease, but also the result of complex cell-extrinsic interactions with host components, including immune cells. For example, effector T lymphocytes and NK cells are thought to participate in an immunosurveillance process which eliminates neoplastic cells, whereas regulatory T lymphocytes and some myeloid cells, including macrophages, can create a milieu that prevents anti-tumor activity, supports tumor growth and reduces survival of the host. Increasing evidence supports the notion that carcinoma cells communicate with immune cells directly, both within and away from the tumor stroma, and that this process fosters suppression of immunosurveillance and promotes tumor outgrowth. An important mode of communication between carcinoma cells and immune cells may involve tumor-derived microvesicles (tMVs), also known as exosomes, ectosomes, or microparticles. These microvesicles carry lipids, proteins, mRNAs and miRNAs, and travel short or long distances to deliver undegraded and undiluted material to other cells. Here we consider the capacity of tMVs to control tumor-associated immune responses, and highlight the known and unknown tMV's actions *in vivo*. We also discuss why microvesicles may play a role in cancer diagnostics and prognostics, and how they could be harnessed for anti-cancer therapy.

Background

A mode of communication between cells in the body is thought to involve extracellular microvesicles (MVs), which incorporate donor cell-derived material (membrane-bound and intracellular) and can be delivered to acceptor/recipient cells. This process, when altered or amplified, is thought to profoundly affect cell biological activities and, consequently, foster pathophysiological processes. Donor and recipient cells may reside in the same microenvironment, in which case MVs regulate local cell-to-cell communication. MVs may also be distributed systemically, for example via lymph and blood vessels (1), and operate as long-range communication signals between organs.

At present, pressing questions include: i) do tMVs target specific components of their immediate micro-environments and do some of these interactions control tumor progression?; ii) which distant organs come in contact with tMVs?; iii) what defines the 'specificity', if any, of tMVs' recipient cells *in vivo*?; iv) do tMVs control host cells that are away from the tumor stroma ? v) what is the relative impact of tMVs on the host response when compared to all other modes of tumor cell/host cell communication?; and vi) can we exploit the accumulating knowledge on tMV biology to identify new vantage points for anti-

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cancer therapy? Some of these questions are being investigated experimentally and are discussed thereafter (see also Fig 1).

MV biogenesis

Extracellular MVs are cell-derived particles that contain a cytosol and are surrounded by a lipid bilayer. Donor cells and their MVs always share the same membrane orientation, though MVs can have different origins (endosomal versus plasma membranes) and vary largely in size (<100nm and >1um). Exosomes (2, 3), typically <100nm in diameter, are MVs that form inside endosomes following intraluminal budding of endosomal membranes. This process creates multi-vesicular bodies that must fuse with the cell surface to secrete their cargo in the extracellular space. Ectosomes (4), typically 100nm–1μm in diameter, are MVs that bud directly from the plasma membrane into the extracellular space. Other MVs have been characterized; they include exosome-like vesicles (5) (<100 nm), which may also bud from the plasma membrane (6), and apoptotic bodies which are produced following cell death (7).

Exosome biogenesis involves the Rab family of small GTPases, which recruit specific effector proteins onto endosomal membranes and drive vesicle docking and fusion (8). Instead, ectosome development depends on arrestins, which promote endocytosis of plasma membrane receptors (9). However, production of both ectosomes (9) and exosomes (10) is thought to require ESCRT (endosomal sorting complex required for transport), a machinery known to be required for sorting of cargo proteins into internal vesicles of multivesicular bodies. There is a stricking convergence between budding of enveloped viruses and MV biogenesis (11).

MV cargo is made of proteins, lipids, mRNAs and microRNAs. The mechanisms that control material inclusion (or exclusion) in MVs remain largely unknown, yet it is well established that different MVs can carry extensively different cargo repertoires. Consequently, MV preparations are often characterized based on the presence (or absence) of molecular pathway components that generate MVs (e.g., Rab27, Tsg101 and Alix) (2), factors produced by MV producing cells (e.g., MHC molecules, CD61 and CD14) (12), proteins involved in target cell selection (e.g., tetraspanins, integrins and selectins) (13), and molecules associated with MVs' biological significance (e.g., Tissue Factor, matrix metalloproteinases, microRNAs) (14).

MV production and release require energy input, RNA synthesis and protein translation (15). The process can be enhanced by exogenous factors including ATP (16), phorbol ester-activated protein kinase C (17), low pH (18), and hypoxic conditions (19), which are all commonly altered in the stroma of growing tumors. However, it remains to be determined whether exosomes and ectosomes have either distinct or overlapping effects on host cell components and tumor development.

tMVs' biological relevance: *in vitro* findings

MV transfer into, and impact on, recipient cells has been mostly analyzed in co-culture systems. These studies have shown that MVs can engage specific receptor/ligand interactions with recipient cells (20–23). MVs can further transfer cell surface receptors (24) and deliver intracellular proteins (25), mRNAs, miRNAs (14, 26, 27) and reporter genes (28, 29) into cells. MVs are thought to change the recipient cells' makeup and thus to influence cellular functions and fate.

The motivation to address whether tMVs affect the immune system comes from experimental and clinical evidence that neoplastic diseases control various immune cell

types (30). Evidence exists that effector T lymphocytes and NK cells can exhibit anti-tumor activity in the tumor stroma; that the presence of tumor-infiltrated T cells increase patients' survival (31); and that regulatory T lymphocytes (Tregs) (32) and myeloid cells, including macrophages (33), however, can generate an immunosuppressive milieu that counteracts anti-tumor immunity, promotes tumor progression and decreases patients' survival. The precise mechanisms of interactions that occur between tumor and immune cells remain largely unknown; nevertheless, recent data suggest that tMVs are involved in promoting tumor outgrowth by controlling the fate of all the immune cell types mentioned above. MVs may induce apoptosis of effector T cells (34–39); switch off NK cell mediated cytotoxicity (40, 41); activate immunosuppressive functions within myeloid cells (21, 42–44); impair dendritic cell production (45); and induce Treg responses (46, 47). Local immunosuppression may also be promoted by extracellular adenosine, which can be released from MVs (48).

In addition to their impact on immune cells MVs may promote tumor outgrowth through other mechanisms, which include degradation of extracellular matrix components (49), acceleration of tumor angiogenesis (29, 50), modulation of stromal cell differentiation (51), transfer of oncogenic activity to other cancer cells (52) and resistance to therapy via sequestration and expulsion of drugs out of tumor cells (53, 54). However, conclusions derived from *in vitro* data alone should be considered with some caution because contacts between MVs and recipient cells in these studies were artificially enforced and the amount of MVs used *in vitro* may be higher than that found *in vivo* (55). The fate of recipient cells *in vivo* may also be dictated by local factors (anatomical features, pH, oxygenation, forces of fluid flow, various cell types and cytokines), which often cannot be reproduced fully *in vitro* (56).

tMVs' biological relevance: analysis in context

Human and mouse carcinomas can produce elevated amounts of MVs. At least some of these vesicles enter circulation (57) and may have biological effects far away from their production sites. Remarkably, Peinado et al. recently reported that mouse bone marrow (BM), which was pre-conditioned with tMVs derived from highly metastatic B16-F10 melanoma cells and then used to reconstitute lethally irradiated subjets, not only promoted tumor infiltration by BM cells, but also accelerated primary and metastatic cancer growth (58). Adoptive tMV transfer experiments further indicated that tMVs could increase vascular permeability at pre-metastatic sites and expand BM progenitors expressing c-Kit, Tie2 and Met. The phenotype of these cells may be functionally relevant because Tie2 can promote tumor angiogenic activity (59), whereas MET is associated with tumor cell invasion (60) and BM cell mobilization (61). Co-culture of tMVs with recipient cells suggested that MET was transferred from tumor cells to bone marrow progenitors via exosomes. Also, reduction of tMV production *in vivo*, through inhibition of *Rab27a* in tumor cells, reduced BM cell recruitment to tumors and delayed tumor outgrowth.

This *in vivo* investigation suggests that tMVs can enhance tumor outgrowth in mice by programming bone marrow progenitor cells with tumor-promoting functions. Nevertheless, the capacity of tMVs to educate BM cells permanently will require further study. It is formally possible that the BM pre-conditioning protocol employed in this study did only skew the hematopoietic repertoire toward the myeloid lineage, which is a process that favors primary and metastatic cancer growth (62, 63). It will also be interesting to define whether tMVs communicate with BM cells through horizontal transfer of information or more simply by surface binding. Finally, *Rab27a* knockdown–mediated inhibition of tMV production also reduced secretion of soluble factors that were previously shown to elicit tumor-promoting host responses (e.g. Ospeopontin (64), PlGF-2 (65, 66) and PDGF-AA

(67)). In general, identifying the relative impact of tMVs and soluble factors (68) as long range signals between tumor cells and BM progenitor cells will require more examination.

A role for tMVs in regulating immune suppression has also been proposed by Chalmin et al. using *in vitro* and *in vivo* approaches (21). In this study, tMVs isolated from different mouse cell lines were shown to enhance the immunosuppressive activity of myeloid cells. The process did not involve horizontal material transfer but instead required direct surface receptor binding between HSP72 on tMVs and TLR2 on myeloid cells. Inhibition of HSP72 expression in tMVs reduced the myeloid cells' capacity to foster metastatic progression. Injections of dimethyl amiloride—used to interfere with tMV secretion *in vivo*—also delayed tumor outgrowth and further enhanced the efficacy of cyclophosphamide therapy in various mouse models (21).

The authors went on to measure the effects of amiloride (an analogue of dimethyl amiloride that is used for the treatment of edema and high blood pressure) in patients suffering from colorectal invasive cancer. Myloid cells prepared from the patients' peripheral blood showed that amiloride treatment decreased suppressor activity (21). These data suggest that interfering with tMV secretion may serve to enhance the efficacy of chemotherapies.

The same study identified that tMV-myeloid cell interaction controlled STAT3 activation and downstream suppressive activities within the sensitized cells. tMVs did not control myeloid cell expansion; this process was instead selectively controlled by tumor-derived soluble factors. Thus MVs and soluble factors may differentially regulate immune cell function and proliferation during tumor progression. Nevertheless adoptive tMV transfer was shown to induce myeloid cell accumulation in the spleen in another study (45), suggesting that tMV's actions may be context-dependent.

It should also be noted that experimental approaches used for *in vivo* studies have limitations. First, the capacity to interfere selectively with tMV production and/or transfer *in vivo* is an unmet need. Diannexin (50), neutral sphingomyelinase 2 inhibitors (69), the H+/Na+ and Na+/Ca2+ channel inhibitor dimethyl amiloride (21), the K+/H+ ATPase inhibitor Omeprazole (70), and the Na(+)/K(+)-ATPase inhibitor Ouabain (71) have been used to control MV biogenesis or binding; however, these agents may also affect non-neoplastic cells. Another challenge imposed by *in vivo* studies is related to difficulties in achieving selective modulation of tMV production or transfer without compromising tumor cell viability. RNAi technology may be used to selectively target tMVs and thus represents a potentially useful tool to establish causal relationships between tMVs and host responses, when properly employed (72). This type of approach should benefit from a better understanding of the molecular players involved in MV biogenesis.

Second, fluorescently labeled tMVs used in adoptive transfer experiments may not fully recapitulate the tropism and impact of endogenous tMVs. Limitations include the existence of various tMV isolation protocols that may enrich vesicles with distinct functions (73); the necessity to transfer MVs as a bolus, which is distinct from uninterrupted tMV production *in vivo*; the fact that MV concentrations observed immediately after transfer may be non-physiological; and the choice of the MV labeling agent. Reagents commonly used to mark MVs are highly lipophilic membrane dyes such as PKH26 (45, 58); these molecules tend to aggregate in micelles, which co-purify with MVs by membrane filtration (100kDa cut off) and ultracentrifugation (unpublished observations) and can contaminate MV preparations. Thus, proper controls should be performed when using membrane dyes *in vivo*. MV marking with membrane-bound fluorescent proteins (e.g., CD63-EGFP (57)), rather than membrane dyes, may allow one to prevent the contamination of MV preparations with unbound fluorescent material, even though the fusion protein may not be present in all MV

types (74). Finally, detection of membrane dyes on recipient cells, either by conventional flow cytometry or immunofluorescence, should not be used to prove transfer of intracellular molecules because MVs may only bind the surface of recipient cells (75). Discrimination between MV surface binding and fusion requires specific experimental settings (76). New technological advances in flow cytometry allow real time imaging at subcellular resolution and may help to discriminate between these possibilities (77). As the details of MV biogenesis become unravelled, new genetic approaches may permit more selective targeting of MV cargo and/or marking of distinct MV types.

Clinical-Translational advances

Role in diagnostics?

Notwithstanding their capacity to control the host response, tMVs may also be relevant for screening asymptomatic patients, diagnosing and profiling disease, and predicting treatment efficacy. At least, initial studies suggest that cancer patients may carry unique circulating MV signatures that reflect the genetic status of the tumor (78). One analysis reported significantly increased exosome levels in lung adenocarcinoma patients when compared to control individuals (79). Another study concluded that circulating tumor-derived (EpCAM+) exosomes in ovarian cancer patients could potentially be used as surrogate diagnostic markers for biopsy profiling (80). Also, some glioblastoma patients were identified with detectable amounts of circulating MVs incorporating a tumor-specific mRNA variant (EGFRvIII) (29), and thus could be diagnosed noninvasively. Interestingly, EGFRvIII mRNA was not detected in serum samples drawn two weeks after resection of the tumor, consistent with this tumor being the source of MVs (29). The diagnostic value of MVs has been investigated in patients with other cancer types, including bladder cancer (81), prostate cancer (82), and colorectal cancer (83). Circulating tumor cells (CTCs) are other relevant candidates for cancer diagnostics, though their low abundance—typically less than one per ml of blood (84)—may render their analysis more challenging.

In some cases MVs may have a prognostic value. A retrospective analysis of stage IV melanoma patients suggested a decreased mortality for those patients who contained protein-poor exosomes in circulation (58). More recently, an analytical technology was reported for MV quantification and protein profiling directly from blood samples (75). The approach consists to introduce MVs onto a portable microfluidic chip for labeling with target-specific magnetic nanoparticles and detection by a miniaturized nuclear magnetic resonance system. The technology was used to screen MVs from glioblastoma patients and thereby predicted which patients would clinically respond to treatment with temozolomide (75). Multiparameter molecular evaluation of MVs should become instrumental in clinical care. Longitudinal analysis makes it possible to monitor tumor molecular responses to therapeutic agents, to determine the emergence of drug resistant tumor variants, and to rapidly phenotype the molecular profile of the emerging cells for adjustment of targeted therapy.

Role in therapy?

More than 10 years ago, MVs isolated from tumor-peptides pulsed, *in vitro* generated dendritic cells were shown to elicit a tumor-specific cytotoxic T cell response that eradicated established, transplanted tumors in mice (12). The same group has reported that vaccination with dendritic cell-derived MVs is a safe approach for cancer patients (85) and new combinations are being tested in clinical trials. *In vitro* manipulation of patient-derived tumor cells could also be employed to load genetically-encoded adjuvants into tMVs, which may then be used for reinfusion into the patient as an anti-tumor vaccine. The presence of bacterial adjuvants, such as flagellin (86), may improve vaccination efficacy. MV removal from the circulation of cancer patients has also been proposed as a therapeutic intervention

(87). Finally, injection of MV biogenesis inhibitors, before or concomitantly with cytotoxic drugs, may increase, at least temporarily, the drug's concentration inside tumor cells. Limiting tMV secretion may also serve to improve anti-tumor immune activity.

Conclusions

Several studies suggest that tMVs control tumor-associated immune responses. The reported presence of circulating tMVs in both humans and mouse models also hints toward an endocrine function for these vesicles, although additional investigation is needed to define their *in vivo* contributions more precisely. tMVs represent interesting vantage points not only for uncovering mechanisms of tumor-host cells interactions but also for developing less invasive diagnostic and prognostic clinical readouts.

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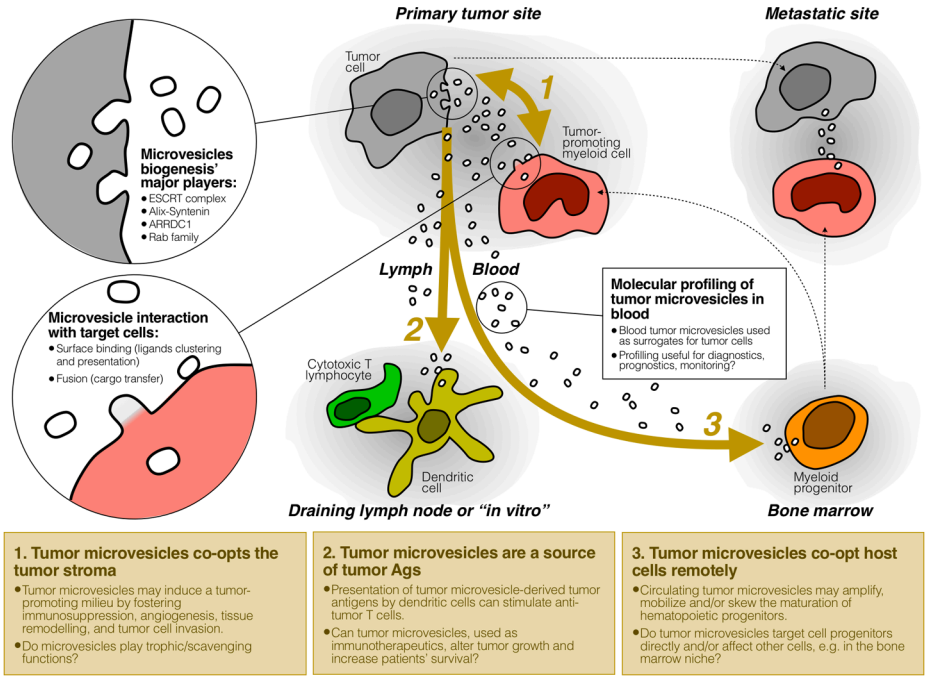

Fig. 1.

A sketch depicting MV tropism *in vivo* in cancer-bearing hosts. 1: Both tumor and host cells produce MVs that may affect other cell types locally, either by surface binding (MVs acting as ligand clustering agents) and/or by transferring bioactive material to target cells (horizontal transfer of proteins, RNAs and lipids). MVs from host cells may provide trophic functions by nurturing tumor cells; 2: tMVs can be drained into lymphatics and shape anti-tumor immune responses; 3: tMVs can circulate to distant organs like bone marrow and alter hematopoiesis. Blood MVs can be harnessed as surrogate tumor cells for diagnostic/prognostic purposes.