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 natural killer 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 antitumor 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 (tMV), also known as exosomes, ectosomes, or microparticles. These microvesicles carry lipids, proteins, mRNAs and microRNAs 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 actions of tMVs in vivo. We also discuss why microvesicles may play a role in cancer diagnostics and prognostics and how they could be harnessed for anticancer therapy.

Microvesicle Biogenesis

Extracellular microvesicles are cell-derived particles that contain a cytosol and are surrounded by a lipid bilayer. Donor cells and their microvesicles always share the same membrane orientation, though microvesicles can have different origins (endosomal vs. plasma membranes) and vary largely in size (<100 nm and >1 μm). Exosomes (2, 3), typically 100 nm or less in diameter, are microvesicles that form inside endosomes following intraluminal budding of endosomal membranes. This process creates multivesicular bodies that must fuse with the cell surface to secrete their cargo in the extracellular space. Ectosomes (4), typically 100 nm to ~1 μm in diameter, are microvesicles that bud directly from the plasma membrane into the extracellular space. Other microvesicles have been characterized; they include exosome-like vesicles (ref. 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 endosomal sorting complex required for transport (ESCRT), a set of machinery known to exploit the accumulating knowledge on tMV biology to identify new vantage points for anticancer therapy? Some of these questions are being investigated experimentally and are discussed thereafter (see also Fig. 1).

Background

A mode of communication between cells in the body is thought to involve extracellular microvesicles, 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 biologic activities and, consequently, foster pathophysiologic processes. Donor and recipient cells may reside in the same microenvironment, in which case microvesicles regulate local cell-to-cell communication. Microvesicles 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 microenvironments 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 tMV 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 with all other modes of tumor cell/host cell communication? (vi) Can we
to be required for sorting of cargo proteins into internal vesicles of multivesicular bodies. There is a striking convergence between budding of enveloped viruses and multivesicular body biogenesis (11).

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

Microvesicle 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 distinct microvesicles (e.g., exosomes vs. ectosomes) have either distinct or overlapping effects on host cell components and tumor development.

The Biologic Relevance of tMVs

**In vitro findings**

Microvesicle transfer into, and impact on, recipient cells has been mostly analyzed in coculture systems. These
studies have shown that microvesicles can engage specific receptor/ligand interactions with recipient cells (20–23). Microvesicles can further transfer cell surface receptors (24) and deliver intracellular proteins (25), mRNAs, miRNAs, (14, 26, 27), and reporter genes (28, 29) into cells. Microvesicles are thought to change the makeup of recipient cells 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 reveals that effector T lymphocytes and NK cells can exhibit antitumor activity in the tumor stroma; that the presence of tumor-infiltrated T cells increases survival of the patients (31); and that regulatory T lymphocytes (Tregs; ref. 32) and myeloid cells, including macrophages (33), however, can generate an immunosuppressive milieu that counteracts antitumor immunity, promotes tumor progression, and decreases survival of the patients. 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. Microvesicles 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 microvesicles (48).

In addition to their impact on immune cells, microvesicles 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 microvesicles and recipient cells in these studies are artificially enforced and the amount of microvesicles 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 (anatomic features, pH, oxygenation, forces of fluid flow, various cell types, and cytokines), which often cannot be reproduced fully in vitro (56).

**Analysis in context**

Human and mouse carcinomas can produce elevated amounts of microvesicles. At least some of these vesicles enter circulation (57) and may have biologic effects far away from their production sites. Remarkably, Peinado and colleagues recently reported that mouse bone marrow, which was preconditioned with tMVs derived from highly metastatic B16-F10 melanoma cells and then used to reconstitute lethally irradiated subjects, not only promoted tumor infiltration by bone marrow cells but also accelerated primary and metastatic cancer growth (58). Adoptive tMV transfer experiments further indicated that tMVs could increase vascular permeability at premetastatic sites and expand bone marrow 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 bone marrow cell mobilization (61). Coculture 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 bone marrow 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 bone marrow cells permanently will require further study. It is formally possible that the bone marrow preconditioning protocol used 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 important to define whether tMVs communicate with bone marrow 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., osteopontin (64), placental growth factor 2 (65, 66), and platelet-derived growth factor (67)]. In general, identifying the relative impact of tMVs and soluble factors (68) as long-range signals between tumor cells and bone marrow progenitor cells will require more examination.

A role for tMVs in regulating immune suppression has also been proposed by Chalmin and colleagues 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 capacity of myeloid cells 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. Myeloid cells prepared from the peripheral blood of these patients 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, microvesicles and soluble factors may differentially regulate immune cell functions 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 the actions of tMVs 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\(^+/\)Ca\(^2+\) channel inhibitor dimethyl amiloride (21), the K\(^+/\)H\(^+\) ATPase inhibitor omeprazole (21), and the Na(+)/K (+)-ATPase inhibitor ouabain (71) have been used to control microvesicle biogenesis or binding; however, these agents may also affect nonneoplastic 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. RNA interference 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 used (72). This type of approach should benefit from a better understanding of the molecular players involved in microvesicle 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 microvesicles as a bolus, which does not recapitulate uninterrupted tMV production by tumors in vivo and likely leads to exceedingly high tMV concentrations immediately after transfer; and the possible impact of microvesicle-labeling agents. Reagents commonly used to mark microvesicles, such as PKH26 (45, 58) are highly lipophilic membrane dyes; these molecules tend to aggregate in micelles, which copurify as PKH26 (45, 58) are highly lipophilic membrane dyes; these molecules tend to aggregate in micelles, which copurify with microvesicles by membrane filtration (100 kDa cut off) and ultracentrifugation (Unpublished observations) and can contaminate microvesicle preparations. Thus, experiments using membrane dye-labeled microvesicles must include proper controls. Microvesicle marking with membrane-bound fluorescent proteins [e.g., CD63-EGFP (57)], rather than membrane dyes, may allow one to prevent the contamination of microvesicle preparations with the unbound fluorescent material even though the fusion protein may not be present in all microvesicle 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 microvesicles may only bind the surface of recipient cells (75). Discrimination between microvesicle 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 microvesicle biogenesis become unraveled, new genetic approaches may permit more selective targeting of microvesicle cargo and/or marking of distinct microvesicle 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 patients with cancer may carry unique circulating microvesicle signatures that reflect the genetic status of the tumor (78). One analysis reported significantly increased exosome levels in patients with lung adenocarcinoma when compared with control individuals (79). Another study concluded that circulating tumor-derived (EpCAM+ ) exosomes in patients with ovarian cancer could potentially be used as surrogate diagnostic markers for biopsy profiling (80). Also, some patients with glioblastoma were identified with detectable amounts of circulating microvesicles incorporating a tumor-specific mRNA variant (EGFRvIII; ref. 29), and thus could be diagnosed noninvasively. Interestingly, EGFRvIII mRNA was not detected in serum samples drawn 2 weeks after resection of the tumor, consistent with this tumor being the source of microvesicles (29). The diagnostic value of microvesicles has been investigated in patients with other cancer types, including bladder cancer (81), prostate cancer (82), and colorectal cancer (83). Circulating tumor cells are also relevant candidates for cancer diagnostics, though their low abundance, typically less than one per milliliter of blood (84), may render their analysis more challenging.

In some cases, microvesicles may have a prognostic value. A retrospective analysis of patients with stage IV melanoma suggested a decreased mortality for those patients who contained protein-poor exosomes in circulation (58). More recently, an analytic technology was reported for microvesicle quantification and protein profiling directly from blood samples (70). This approach introduces microvesicles 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 microvesicles from patients with glioblastoma and thereby predicted which patients would clinically respond to treatment with temozolomide (70). Multiparameter molecular evaluation of microvesicles 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, microvesicles isolated from tumor-peptide 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 microvesicles is a safe approach for patients with cancer (85), and new combinations are being tested in clinical trials. In vitro manipulation of patient-derived tumor cells could also be used to load genetically encoded adjuvants into tMVs, which may then be used for reinfusion into the patient as an antitumor vaccine. The presence of bacterial adjuvants, such as flagellin (86), may improve vaccination efficacy. Microvesicle removal from the circulation of patients with cancer has also been proposed as a therapeutic intervention (87). Finally, injection of microvesicle biogenesis inhibitors before or concomitantly with cytotoxic drugs may increase, at least temporarily, the concentration of the drugs inside tumor cells. Limiting tMV secretion may also serve to improve antitumor immune activity.

Conclusions

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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.J. Pittet

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