



Cancer Cells Shed Micro-Vesicles from Actin Stress Fibres - a New Cooperative Mechanism for Metastasis?

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Introduction

Since decades cancer therapy has made slow but continuous strides in fighting primary tumors, but progress in the suppression of metastasis remains elusive. The spread of cancer to distant locations in the body is the major cause of cancer morbidity and mortality, and accounts for about 90% of cancer deaths. Although cancer survival rate has been significantly improved over the years, the improvement is primarily due to early diagnosis and cancer growth inhibition (Guan X, 2015) [1].

Maybe the best known and most common form of metastasis is the movement of cancer cells across morphologic boundaries in their quest to find better growth conditions -and of course to escape or hide from the immune system. For example, before wandering to distant parts of the body, many cancer cells are known to develop tiny “feet”, so called invadopodia. Using a high-resolution time-lapse intravital imaging approach, Leong HS et al. (2014) [2] visualized the dynamics of cancer cell extravasation in vivo. The researchers watched cancer cells trying to escape from the endothelial junction’s bloodstream of chicken embryos and mice to establish themselves in a new location in the stroma. Those cells lifted themselves out of the blood stream, with their invadopodium forming tentacles which pried open a spot between two blood vessels.

This situation -i.e. that cancer cells try to metastasize away from adversarial conditions (low oxygen, circulating immune cells in the bloodstream) already suggests that metastasis is not just an intrinsic property of cancer cells but may also be simply their stress “response” to get away from trouble. This begs the question, what is not more stress than being attacked by chemotherapy? And indeed, recent experimental studies in mice suggest that chemotherapy itself can exacerbate metastasis. How can that be? Keklikoglou I et al. (2018) [3] found that two classes of cytotoxic drugs broadly employed in pre-operative (neoadjuvant) breast cancer therapy, taxanes and anthracyclines, can exacerbate metastasis concomitant

with the changes in the number and the bio-chemical make up of extracellular vesicles (ECVs). Her findings indicate two problems at once: Not only can metastasis be supported by the very method which is designed to fight malignant tumors, but as well this could be via other mechanisms than merely the physical translocation of cancer cells across space and time or can it?

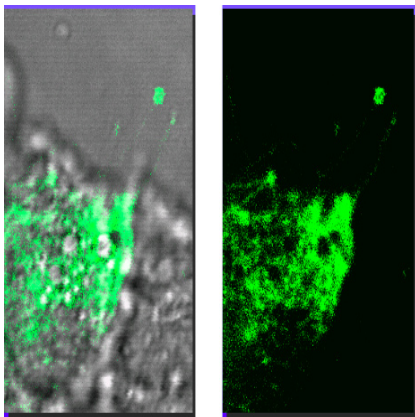
Researchers have since long been on the lookout for other means of transportation of cancerous information or its physical entities from the primary tumor to distant parts in the body. A possible candidate has occurred over the last years - “extracellular vesicles” (EVs), including exosomes,” that can facilitate the seeding and growth of metastatic cancer cells in distant organs. Whether released from the cell interior or the plasma membrane, it has been known for longer time that tumors release such particles. A big hindrance to research has always been the purification of such particles. Novel ways of doing it have been designed by Yeo T et al. (2016) [4], but their upscaling remains to be seen. For addressing novel ways of fighting cancer and modulate, adjust and improve chemotherapy, it is of utmost importance to be able to identify the origin of so called extracellular vesicles. Do they come from the cell interior -in form of exosomes, vesicles- or from the plasma membrane (in form of ectosomes, microvesicles)? Depending on the origin we could then address the biochemical machinery within those lipid particles and mechanisms of their well-known metastatic potential. Being one of the first researchers who created such particles in the lab, I report here findings which are borne out of our efforts by Pick et al. (2003) [5] to investigate the distribution and function of heterologous expressed receptors on the cell surface of immortal cell lines. My findings have stark implications for cancer research: we are able to generate labeled microvesicles from an immortal cell line, and identify their origin from structures resembling cancer cell filopodia, potentially pointing to a link between invadopodia and the generation of such particles which have been reported to have metastatic potency.

Methods

The methods have been published in detail elsewhere (Pick et al, 2003) [5] (EP patent #1356291, Vogel et al, 2001) (US20080139403A1, Vogel et al 2008) [6]. In short, we used laser scanning confocal microscopy to visualize extracellular vesicles and their generation from filopodia resembling actin stress fibers. Cells were visualized using the DIC transmission of system. Fluorescent membrane labeling allowed us to visualize the cells and the microvesicles generated from them in the fluorescent channel. Ca²⁺ dye loading was used to visualize the intact biochemical machinery of the vesicles insofar that vesicles still contained the esterase's to load the dye correctly and could indicated Ca²⁺ levels correctly when exposed to 5HT3 stimulation or ionophore (results not shown in this mini review, separate manuscript in preparation).

To be able to have a high receptor density of 12 000 receptors/ μm^2 aggregating on membrane patches on living cells, I brought a patented transfection method (US 5593875A) pioneered by Martin and Wurm (1997) [7] to Horst Vogel's lab at EPFL. A fluorescent receptor ligand (specifically designed by Glaxo for this purpose) was used to visualize the heterologously expressed 5-HT3 receptor and its functionality in both the transfected cells and their derived microvesicles. Confocal time lapse was used to show vesicle ejection from the transfected cells, with the pinhole adjusted to manipulate confocality or weaken it. Cytochalasin B or D (Sigma-Aldrich) (stock solution with a concentration of 2 mg/ml in DMSO) was added to the preheated DMEM medium in a final concentration of 10 to 20 $\mu\text{g}/\text{ml}$. The filopodium like budding and its vesiculation are dependent on the cell age and cell line and lasted between 5 and 60 minutes.

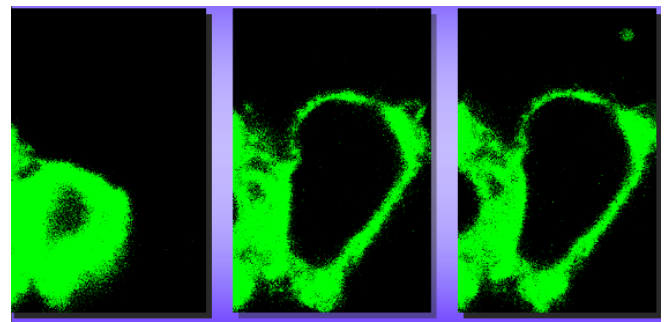
Results (Figure 1)



Pictograph 1 shows a HEK cell whose heterologously over expressed membrane receptor (here: 5-HT3) is la-beled with a fluorescent extracellular specific ligand [8,9]. We used a powerful, patented method to transfect those cells (expired patent by

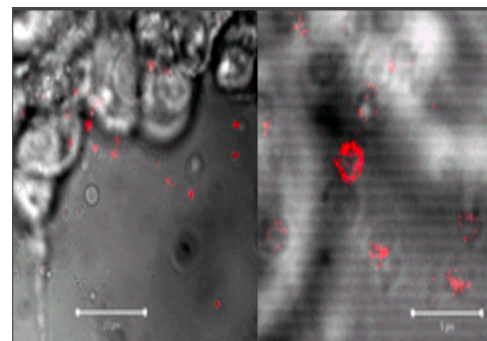
Wurm F and Martin J, 1997) [7] to be able to reach high receptor densities (more than 10^7 receptors/cell). As can be clearly seen the microvesicles originated from the plasma membrane and are released by elongated filopodium like processes which are reported to form under hypoxia like conditions and here form as response to cytochalasin B. I investigated numerous cells, and more than 80 percent of vesicles originate from those filopodium like protrusions. The advantage of this cell system is that we can avoid the term extracellular vesicle (EV) and clearly state that our particles are microvesicles. Scale bar in relation to vesicle size please see Figure 3.

(Figure 2)



Pictograph 2 shows one of those rarer events where a vesicle is released from a non-protruding part of the cell. Like in Figure 1 the cell is labeled with 5ht3 receptor ligand. The image sequence shows the speed of the release (left, 12min after Cytochalasin addition, center, 1 minute later and right, 3 minutes after that). In general, after application of cytochalasin, the cells adapted a round form. Microfilaments contract and condense to local aggregates in the cell cortex. The otherwise continuous actin cytoskeleton became fragmentary. Supported by the cytoplasmic pressure, this effect leads to an expansion of the endoplasm in these regions. As a result, bud-ding of the cell membrane occurs at these locations. These buds are either bulbous (as shown here) or -in most cases- pedunculate (See Figure1) in form. Again, for scale comparison, see Figure 3.

(Figure 3)



Pictograph 3 shows size and intensity of ligand labeling of those vesicles exposed for 70 s to the receptor ligand (labeling can clearly be seen after 15 s, not shown here). The size of the vesicle is directly in the confocal plane and therefore can be assessed by the scale bar on the right panel (1 micro m). Scale bar is (20 micro m left, 1 um right)

Conclusion

Our results open the unexpected avenue that filopodia (so called invadopodia) might be not only the feet cancer cells use to move from their origin to distant locations in the body, they might actually convey their metastatic property as well by shedding microvesicles. In this way, metastasis might be just two sides of the same coin: Invadopodes translocate cancer cells and at the same time shed vesicles which have been associated by numerous investigators with metastatic processes.

Our results show the release of microvesicles from the plasma membrane of physiologically compromised immortal cells. Please note that the cytochalasin dosage we used was not discriminate enough to differentiate between glucose transport inhibition and cytochalasin B's action on the actin structure. Further research is needed to verify whether those protrusions are same as or similar to invadopodia as formed by cancer cells under hypoxic or immune cell rich environment exposed conditions (HS Leong et al, 2014) [2]. As we pointed out in the introduction, such invadopodia are used as escape route for primary tumors from their original location. Any block by cytochalasin of the glucose transporter could mimic such adversarial conditions. Yet, cytochalasin is known to affect the actin filaments already from a concentration of 2 μ M on, lower than the one we used (20-40 μ M).

The elegance of our study is that we can mimic stress conditions for cancer cells *in vitro* in a way which allows to verify the identity of their released particles and their origin from the cell membrane. As summarized by Clancy JW and D'Souza-Schorey C (2012) [10], recent advances in the study of tumor-derived microvesicles reveal new insights into the cellular basis of disease progression and the potential to translate this knowledge into innovative approaches for cancer diagnostics and personalized therapy. They have been thought to deposit paracrine information and create paths of least resistance, as well as be taken up by cells in the tumor micro-environment to modulate the molecular makeup and behavior of recipient cells. The complexity of their bioactive cargo-which includes proteins, RNA, microRNA, and DNA-suggests multipronged mechanisms by which microvesicles can condition the extracellular milieu to facilitate disease progression.

The formation of these shed vesicles likely involves both a redistribution of surface lipids and the vertical trafficking of cargo to sites of microvesicle biogenesis at the cell surface. We want to point here multifold effect of extracellular vesicles (which

include microvesicles and exosomes) that promotes metastasis by a) their physical translocation b) triggering molecular events supporting metastasis and/or c) transforming target cells by fusion / take up. Our model system is perfectly suited to investigate such processes *in vitro* and should be of interest to labs which do care about biological effects and cancer pathogenesis. We are currently seeking collaboration with laboratories which have FLIM microscopy capabilities.

We want to point out recent results published by Gomzikova M et al (2017) [11] that cytochalasin B-induced membrane vesicles convey angiogenic activity of parental cells. Brilliantly written, this paper brings to mind that cancer cells not only might metastasize away from challenging microenvironment, but bring a positive environment to them. Instead of escaping from hypoxic conditions they can bring oxygen supply to them in form of angiogenesis.

DePalma's group has shown that two breast cancer antagonists have resulted in increasing metastasis concomitant with EV release (Keklikoglou I, et al, 2018) [3]. Yet the group was not able to definitely say what type of EVs were released. As there are reports that EVs can also inhibit cancer, the situation is complex (Al-Nedawi K, 2014). We might speculate that the anti- or prometastatic effect of EVs depends on whether microvesicles or ectosomes have been investigated in those experiments, and how much cross contamination between both has occurred. Of course, the anti- or prometastatic potency of various systems will depend on the type of cancer and type of chemotherapeutics used, among other conditions. Please note that signal molecule location can be tricky - for example it has been shown RNA can be located on the exterior of peroxisomes via the noncovalent contacts with the membrane proteins (Yarmishyn A et al, 2016) [12].

An obvious and crucial mechanism for extracellular vesicles to facilitate metastasis would be, of course, their direct interaction with target "host" cells. As mentioned above, a row of live microscopic techniques is required to touch on that question. As mentioned above, we recommend FLIM microscopy -and FRET- but other powerful tools have indeed been employed by other labs. They involve live or cryo-light microscopy, and powerful electron microscopic techniques. For example, B16 BL6 tumors in mice have shown to accelerate their growth by uptake of their own exosomes, and their uptake was demonstrated by confocal microscopy of showing the fluorescent PKH26 signal spread within the cells after 24 hours in comparison to 4hrs incubation (Matsumoto, et al. 2017) [13]. Please be aware that these experiments used exosomes and not microvesicles. Electron microscopy was used to show the size of the exosomes. The role of microvesicles in targeting host tissue remains more obscured. Their importance in metastasis facilitation, however, has triggered increasing interest by electron microscopy groups as well, moving from architecture and their release mechanism from the plasma membrane towards the

analysis of their direct interaction with the target tissue. In this respect Nanou A, et al (2018) [14] show exciting SEM images of circulating tumor cells and tumor derived extracellular vesicles on the surface of unidentified human cells. More work remains to be done to characterize the nature of such extracellular vesicles.

Arusu et al (2019) employed correlative light and electron microscopy and showed that extracellular vesicles (EV) with GFP tagged plasma membrane protein, HAS3, were indeed lying on the recipient cell's plasma membrane, while the level of EV-derived intracellular signal was low. Immunoelectron microscopy supported this finding. Furthermore, hyaluronan oligosaccharides decreased the numbers of bound EVs, suggesting that CD44 (a surface protein) participates in the regulation of their binding. We agree with their assessment that it will be up to live cell imaging at high resolution to obtain definite answers on the detailed mechanisms of binding, fusion and endocytosis of EVs. It is interesting that this group remains cautious and does refrain from calling their EVs microvesicles, despite them being labeled with GFP tagged plasma membrane protein, HAS3.

A step further towards the identification of EVs venture Antonyak, et al. (2018) [15]. Using SEM and fluorescence microscopy they show that microvesicles shed from the surface membrane of two different human cancer cells, MDAMB231 breast carcinoma cells and U87 glioma cells, are capable of conferring onto normal fibroblasts and epithelial cells the transformed characteristics of cancer cells (e.g., anchorage-independent growth and enhanced survival capability) and that this effect requires the transfer of the protein cross-linking enzyme Tissue Transglutaminase (TTG) cooperating with fibronectin.

Clearly ultrahigh resolution live confocal microscopy such as STED or particle membrane interaction observing FLIM, FRET is needed to address the precise mechanism of how and under which conditions individual microvesicles contribute to metastasis. Again, our system is superbly designed to address this question. Last not least, it is no surprise that cytochalasin has a unique antineoplastic activity that could potentiate a novel class of chemotherapeutic agents (Trendowski, et al. 2015) [16]. This underlines the importance of our work: We use cytochalasin to generate microvesicles from stress fibers, yet cytochalasin itself is a rather "benign" agent. Its arrest of the actin filament system may inhibit tumor growth but actually facilitate metastasis. As mentioned at the begin of our discussion, further research has to be done to differentiate between cytochalasin's glucose transporter inhibition and its ability to affect the actin system and their probable cross talk. In this respect it should be mentioned that cytochalasin, depending on concentration, can have a reversible effect on cell actin filament organization. While a definite proof of the cooperativity -or complicity- of microvesicles and invadopodia or stress fibers for the facilitation of metastasis was beyond

the scope of our work, we have certainly opened the avenue for that possibility.

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